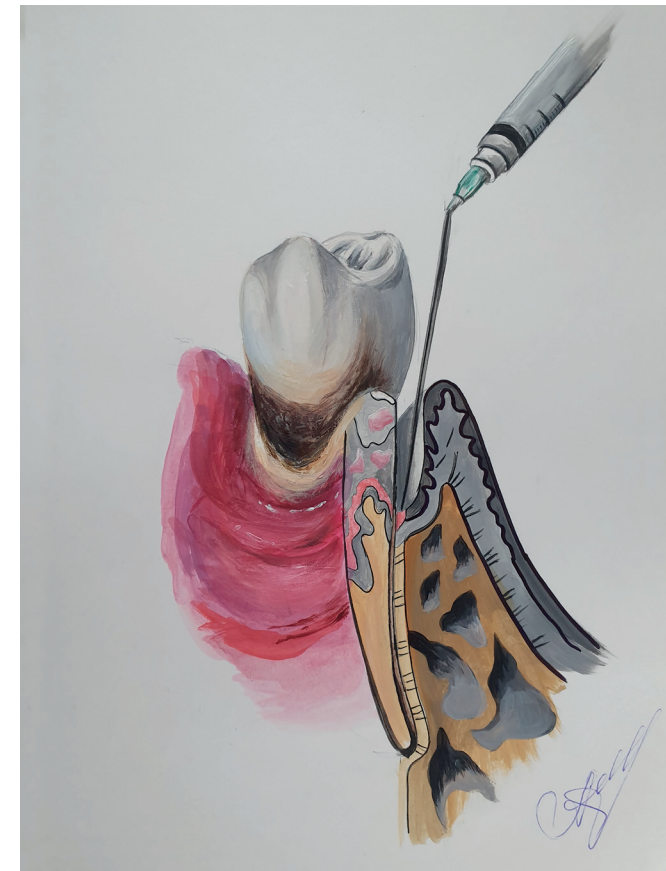


Clinical, Microbiological and Immunological Effects of Antiseptics in Periodontal Treatment



Anton Vitt

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Anton Vitt



**Karolinska
Institutet**

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Clinical, microbiological and immunological effects of antiseptics in periodontal treatment

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To my family

ABSTRACT

Periodontal diseases are characterized by high prevalence in many populations and the chronic and progressive course of the disease. A fundamental stage of treatment is mechanical debridement of the tooth surface. During debridement antimicrobial agents may be applied as adjunctive intrasulcular irrigants.

There is lack of consensus as to the optimal type of antimicrobial therapy in treatment of periodontal disease. A new antiseptic solution, containing polyhexamethylene guanidine phosphate (PHMG-P) as the active ingredient, has recently been marketed as an adjunctive irrigant in periodontal treatment. Since there is no sufficient history of oral application of this antiseptic, few scientific is evidence available to support its efficiency.

The aim of the thesis was to investigate the effects of antiseptics on bacteria and human cells and to evaluate the efficacy of adjunctive (PHMG-P) irrigation of periodontal pockets during scaling and root planing (SRP).

Clinical, microbiological and immunological parameters were evaluated.

In the initial *in vitro* study (*Paper I*), the anti-microbial activity of antiseptics was investigated. Using a quantitative suspension method, PHMG-P was compared with chlorhexidine (CHX) on standard quality control bacterial strains of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*, the periopathogens *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* and also *Streptococcus mutans*, and *Lactobacillus acidophilus*, microorganisms implicated in dental caries. In *Paper II* the cytotoxic and immunomodulatory effects of the antiseptics on human gingival fibroblasts were determined. In *Paper III* the clinical efficacy of intrasulcular irrigation with PHMG-P and CHX as adjunctives to periodontal debridement in patients with severe chronic periodontitis was investigated. Finally, *Paper IV* evaluated the impact of the antiseptics on microflora and cytokine profiles in periodontal pockets undergoing treatment.

Papers I and II showed that PHMG-P had anti-microbial activity and cytotoxicity and expressed anti-inflammatory properties. In *Paper III* it was shown that adjunctive PHMG-P irrigation improved the short-term outcome of SRP, but had no significant long-term effect on the mean pocket depth. *Paper IV* showed that although antiseptic treatment caused changes in biomarkers and bacterial counts, no differences in these parameters were observed between the treatment groups at any of the follow-up examinations.

Conclusion: In the *in vitro* studies, PHMG-P showed pronounced antimicrobial activity. The primary response to periodontal treatment was attributable to the mechanical debridement. The adjunctive antiseptic irrigants, applied in accordance with the study protocol, had only limited effects.

Key words: Periodontal disease; periodontitis; periodontal treatment; antiseptics; chlorhexidine; polyhexamethylene guanidine phosphate; fibroblasts; cytokines; prostaglandin E2; matrix metalloproteinase; inflammation; periopathogens.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-IV).

- I. Vitt A, Sofrata A, Slizen V, Sugars R V, Gustafsson A, Gudkova E I, Kazeko L A, Ramberg P and Buhlin K (2015) Antimicrobial activity of polyhexamethylene guanidine phosphate in comparison to chlorhexidine using the quantitative suspension method, *Annals of Clinical Microbiology and Antimicrobials* 14:36, DOI 10.1186/s12941-015-0097-x
- II. Vitt A, Slizen V, Boström E, Yucel-Lindberg T, Kats A, Sugars R V, Gustafsson A & Buhlin K (2017) Effects of polyhexamethylene guanidine phosphate on human gingival fibroblasts, *Acta Odontologica Scandinavica*, 75:7, 524-529, DOI: 10.1080/00016357.2017.1350993.
- III. Vitt A, Gustafsson A, Ramberg P, Slizen V, Kazeko L A & Buhlin K (2019) Polyhexamethylene guanidine phosphate irrigation as an adjunctive to scaling and root planing in the treatment of chronic periodontitis, *Acta Odontologica Scandinavica*, DOI: 10.1080/00016357.2018.1541099
- IV. Vitt A, Sorsa T, Lira Junior R, Babenka A, Boström E, Slizen E, Gustafsson A & Buhlin K Adjunctive antiseptic irrigation of periodontal pockets: effects on microbial and cytokine profiles. *Manuscript*.

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LIST OF ABBREVIATIONS

BOP	Bleeding on probing
CHX	Chlorhexidine
DMEM	Dulbeco's Modified Eagle's
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
GCF	Gingival crevicular fluid
HGF	Human gingival fibroblasts
IL-1 β	Interleukin 1 beta
MMP	Matrix metalloproteinase
MTT	Dimethyl thiazolyl diphenyl tetrazolium bromide
OD	Optical density
PBS	Phosphate-buffered saline
PHMG	Polyhexamethylene guanidine
PHMG-H	Polyhexamethylene guanidine hydrochloride
PHMG-P	Polyhexamethylene guanidine phosphate
PI	Plaque index
PPD	Pocket probing depth
RANKL	Receptor activator of nuclear factor-kB ligand
RT-qPCR	Real-time quantitative polymerase chain reaction
SRP	Scaling and root planing

1 INTRODUCTION

1.1 Periodontal diseases

The periodontium comprises the tissues supporting the tooth: the gingiva, the periodontal ligament, the alveolar bone and the root cementum. Of all the periodontal pathologies, the most widespread are inflammatory conditions, such as gingivitis and periodontitis (1). While gingivitis is confined to the gingiva, periodontitis involves all the periodontal tissues. The main feature of periodontitis is breakdown of the junctional epithelium. However, it is not always easy to distinguish clinically between gingivitis and slight periodontitis, i.e. to establish the fact whether the dentogingival junction is breached.

1.1.1 Periodontitis

Periodontitis is defined as inflammation of the periodontal tissues, caused by interaction of the oral biofilm with the host immune system and manifested as disruption of the dento-gingival junction. If untreated, the condition progresses to tooth loss. Periodontitis also affects general health by increasing the risk of myocardial infarction and has a negative effect on quality of life (2-4). In some populations periodontal diseases are a grave public health challenge (5). In the classification by Armitage in 1999, chronic periodontitis was denoted as generalized or localized (6). Severity was further denoted as slight, moderate or severe. Recently a new system has been proposed, classifying periodontitis in terms of stages and grades, adding new headings for peri-implant conditions and gingival health on intact and reduced periodontium (1).

1.1.2 Dental plaque

The early nonspecific plaque hypothesis postulated that periodontal inflammation was induced in response to plaque accumulation (7). Later the specific plaque concept presumed implication of certain microorganisms as causative agents of the disease (8). The evidence supporting this concept included the elevated levels of putative periopathogens in periodontal lesions, the resolution of inflammation after elimination of these microbes and induction of the disease in gnotobiotic rats by introduction of specific bacterial species (9, 10). The role of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Tannerella forsythia* was generally accepted and they were regarded as “consensus” periodontopathogens. *Eubacterium nodatum*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Prevotella nigrescens* and *Treponema denticola* are also strongly associated with chronic periodontitis (11). More recently the biofilm has been recognized as a significant risk factor for periodontal diseases. The “ecological plaque hypothesis” was advanced (12). It formulated that the imbalance in non-pathogenic biofilm

auto-regulation, behavioral aspects and the host immune response maximizes the deleterious impact of dental plaque leading to accumulation of periopathogens and enhances the risk of periodontal disease.

The biofilm incorporates single cells and microcolonies in a highly hydrated exopolymer matrix (13). These entrapped cells behave differently from their free-floating counterparts. A general characteristic of biofilm communities is that compared with their planktonic counterparts, they tend to be considerably more resistant to antibiotics and antimicrobial substances, including those generated by the host defense mechanisms (14, 15). The pathogenic effects of the dental biofilm can be moderated by alleviating the microbial burden and effectively maintaining normal oral biocenosis via routine hygiene and treatment procedures (16, 17).

1.1.3 Immune response

Periodontitis is initiated by microbial plaque, but progression of the disease is closely associated with the host immune response (18). Host inflammatory mediators play important roles in the control of periodontal infection, yet they are also implicated in tissue destruction (19, 20). Cytokines are soluble low molecular weight proteins able to bind to specific receptors, initiate intra-cellular signaling cascades and regulate the range and duration of the inflammatory response (21, 22). They are produced by epithelial cells, fibroblasts and phagocytes (neutrophils and macrophages) in the acute phase of inflammation, and by immune cells (lymphocytes) in the adaptive immune response (23).

The innate host immune system initially recognizes microbial components via pattern recognition receptors such as toll-like receptors (TLR)-2, TLR-4 and nucleotide-binding oligomerization domain receptors (24). The binding of microbial components to these receptors, through a series of intracellular biochemical signals, results in production of pro-inflammatory cytokines and chemokines, including tumor necrosis factor (TNF)- α and interleukin (IL)-1 β , which in turn recruit leukocytes to the periodontal space to combat the infection (19). Subsequently leukocytes generate substantial amounts of inflammatory cytokines such as TNF- α , IL-1 β and IL-6. Production of receptor activator of nuclear factor-kB ligand (RANKL) disrupts the balance in the level of osteoclast differentiation factor, causing osteoclastogenesis, ultimately resulting in increased resorption of alveolar bone (25). In addition to direct promotion of bone resorption, innate immunity cytokines also inhibit osteogenic differentiation (26). The local chronic inflammatory reaction causes a pronounced change in the ratio of matrix metalloproteinases (MMP) to tissue inhibitors of metalloproteinases (TIMP) and this is responsible for decompensation of extracellular matrix of the connective tissues. Activated by antigen-presenting cells, the adaptive immune system (T and B cells) also contrib-

utes to tissue resorption. For example, the ability of Th17 cells to produce IL-6 and to up-regulate IL-1 β and TNF- α synthesis may increase expression of MMP and RANKL (27). Studies have also demonstrated that B-cells produce RANKL in response to stimulation by periodontal pathogens (28).

In contrast to the destructive pathway involving primarily pro-inflammatory cytokines, regulatory pathways mediated by the anti-inflammatory cytokines IL-10, IL-4 can control or attenuate disease progression (29, 30). On the one hand, pro-inflammatory cytokines play an essential role in clearing infection. On the other hand, they play destructive roles in the periodontal environment.

Prostaglandins comprise a group of potent inflammatory mediators derived from arachidonic acid and induce a wide variety of biological responses (31). They influence vasodilatation, vascular permeability, oedema, pain and fever, and also play some part in neutrophil and monocyte chemotaxis (32). Prostaglandin (PG) E₂ is one of the most prominent agents in the pathogenesis of periodontitis (33). It stimulates production of inflammatory mediators and MMP, as well as osteoclast formation via RANKL.

Antiseptics may reduce the bacterial burden by antimicrobial action, which in turn suppresses inflammation by reducing the amount of pro-inflammatory cytokines to the level at which they no longer contribute to progression of the disease.

In the context of current theories on the aetiology of periodontal disease, a useful adjunct to treatment would be an anti-inflammatory antiseptic, combining both bactericidal activity against periopathogens and the capacity to arrest periodontal tissue disintegration.

1.2 Association between periodontal diseases and systemic diseases

Many investigators have reported associations between periodontal and systemic diseases (34, 35). However, the underlying mechanisms remain unclear. There are two well-defined interaction mechanisms. Firstly, patients with periodontitis may experience more frequent and severe bacteremia, which in turn leads to exposure of internal organs to oral bacteria or their by-products. Once bacteria reach the target organ, they can elicit an inflammatory response. The second putative mechanism involves cytokines generated by inflamed periodontal tissues. These cytokines enter the circulatory system and are transported to other organs where they induce an inflammatory response.

A relationship between periodontitis and cardiovascular diseases has been established (35). Trials showed strong evidence that periodontal treatment mitigated the risk of cardiovascular diseases (36). A close association was also established between diabetes and periodontitis, but longitudinal clinical studies, animal models and cell/tissue *in vitro* experiments are required to ascertain bidirectional mechanisms interrelating both pathologies (37).

Chronic periodontitis may also be correlated with other diseases. Several reports link periodontitis and chronic obstructive pulmonary disease, pneumonia, chronic kidney disease, rheumatoid arthritis, cognitive impairment, obesity, metabolic syndrome and cancer (38). Cohort studies indicate that periodontal disease is associated with an increased risk of premature death (39).

1.3 Periodontal treatment

The aim of periodontitis management is to arrest inflammation, prevent contamination of underlying tissues, and create conditions for healing and regeneration of periodontium.

The treatment generally divided into several phases: emergency, nonsurgical, surgical, restorative and maintenance, which are not strictly successive. Maintenance care, for instance, is usually an intermission in between nonsurgical, surgical and restorative phases and it is routinely repeated during lifetime after successful treatment. Surgical or restorative phase could be omitted, if redundant. Traditional mechanical methods involving scaling and root planing (SRP) are non-specific in nature and envisage scrupulous debridement. SRP could be performed either gradually quadrant by quadrant with one-two week intervals or on a full-scale within 24 h. The chance that untreated pockets may be a source of recolonization for the treated sites substantiated the need of full mouth debridement (40). Better clinical and microbiological outcomes of periodontal treatment using full mouth disinfection have been reported (41). However, other research groups showing similar efficiency of both approaches, failed to confirm the superiority of full-mouth disinfection (42, 43). Gingival curettage, i.e. removal of pocket lining adjunctional epithelium was designed to promote attachment of new connective tissue to the tooth, provides no additional benefit when compared to SRP. American Dental Association did not recommend gingival curettage as a method of treatment and regarded it as a procedure of no clinical value (44).

Rough tooth surface is known to promote plaque formation (45, 46). In this regard should the SRP include intentional removal of root cementum? Dental calculus disposal implies elimination of root cementum. In the same time excessive cement removal is not obligatory to get rid of endotoxins and hence should be avoided (47).

Mechanical debridement is laborious and time-consuming and might be ineffective for eliminating pathogenic bacteria from periodontal pockets (48). Since microbial plaque is strongly implicated in periodontitis, an indispensable step in the treatment protocol is to reduce the burden of periopathogens. Even after meticulous eradication of dental plaque some subgingival microorganisms persist (49). It is difficult to reach the base of pockets with curettes and the rough surface of the root may hinder complete removal of subgingival plaque. Studies have shown that failure of chronic lesions to heal may be due to insufficient eradication of pathogens (50). After SRP, anti-infective drugs may help reduce the number of periopathogenic and other microbial species in the biofilm.

1.3.1 Antibiotics

Systemic antibiotics may be administered during treatment of chronic periodontitis, to promote tissue healing following SRP (51). Systemic administration of metronidazole and amoxicillin improves clinical outcome of non-surgical periodontal treatment and reduces further surgical treatment needs (52). However, the undesirable side effects, including hypersensitivity, gastrointestinal disturbances and especially the development of bacterial resistance should be taken into account and antibiotics should be prescribed restrictively (53). To enhance the efficacy of periodontal treatment and to circumvent the undesirable effects of systemic antibiotics, locally delivered antimicrobials have been proposed for anti-infective management of periodontal diseases to modify biofilm composition and to re-establish its autoregulation.

1.3.2 Antiseptics

Antiseptics constitute a diverse class of antimicrobial drugs applied to skin or mucous membranes to treat or prevent infection. Their administration includes, but is not restricted to preconditioning of the skin surface prior to intervention, treatment of wounds and routine disinfection of the mouth as part of an oral hygiene program. Intraorally, anti-infective drugs may be delivered by rinsing, by irrigation, by subgingival irrigation with syringes, or by power devices. Biocides show a broader spectrum of activity than antibiotics and prefer specific intracellular targets whereas antiseptics may have multiple targets (54).

1.3.3 Mouthrinses

The first mention of mouthrinses in Chinese medical practice dates back to around 2700 B.C. (55, 56). Even without reliable scientific evidence, our ancestors resorted to cleaning their teeth (57). Many oral hygiene aids, like chewing sticks, tooth powder and home-made mouthrinses were used.

In 1879 Joseph Lawrence and Jordan Wheat Lambert created a mouthwash and named it Listerine® to commemorate Joseph Lister, known as the father of aseptic surgery (58).

Nowadays various mouthwashes are available. Active ingredients include alcohol, fluoride, antibacterial enzymes, essential oils, cetylpyridinium chloride and hydrogen peroxide. However, a mouthwash cannot substitute regular oral hygiene self-care, such as tooth brushing and flossing. It is doubtful whether a healthy person needs anti-bacterial mouthwashes to complement daily oral hygiene routine.

1.3.4 Biguanides

The first chlorhexidine (CHX) preparation was synthesized and marketed in the United Kingdom in early fifties. It exhibits a potent antibacterial action against a wide range of both Gram-positive and Gram-negative bacteria (59). Twenty years later CHX found wide use in dentistry, gynecology, urology, ophthalmology and to date it is regarded as the gold standard among antiseptics (60, 61).

Antimicrobial action of CHX is based on its ability to alter the integrity of the bacterial inner membrane, leading to increased permeability and leakage of intracellular ions (62). Some adverse effects of CHX include staining of the teeth and tongue, distorted taste sensation and desquamation of the oral mucosa (63). Aside from its antibacterial properties, CHX is toxic to many mammalian tissues and cells: sperm, macrophages, skin epithelium etc. Studies have shown that chlorhexidine induces a dose-dependent inhibition of cellular proliferation and reduces the ability of fibroblasts to produce collagen and non-collagen proteins (63). A more cautious approach to its use in oral surgery was advised because of possible adverse effects on periodontal tissue (63, 64).

Contradictory research results were obtained following application of antiseptics in periodontal treatment. Most studies confirm that CHX solution as an adjuvant to SRP does not provide additional benefit in terms of improving the clinical outcome of periodontal treatment (48, 65). However, M. Guarnelli et al. 2008 demonstrated beneficial effect of CHX in ultrasonic instrumentation of patients suffering from aggressive periodontitis (66). Bacteremic episodes, often induced by SRP, are frequently associated with chronic periodontitis. Under these circumstances, antiseptics may decrease the release of bacteria into the blood stream (67). Subgingival instrumentation with concomitant povidone-iodine rinsing has been shown to mitigate the risk of development of oral bacteremia (68).

Polyhexamethylene guanidine (PHMG) derivatives are members of the polymeric guanidine family, widely used for many years as antiseptics in medicine and the food industry (69). PHMB interacts with acidic, negatively charged phospholipids in the bacterial membrane, causing increased fluidity, permeability and loss of integrity, followed by the death of the cell (70, 71). It was also found that PHMB could bind to nucleic acids (72). Structurally similar conjugates such as polyhexamethylene biguanide hydrochloride (PHMB-H) and polyhexamethylene guanidine phosphate (PHMG-P) are synthesized by incorporating different anions into the PHMG molecule. PHMB-H has been extensively tested *in vivo* and *in vitro*. In the form of a mouthwash, PHMB-H consistently inhibited plaque regrowth and reduced oral bacterial counts, indicating its potential role as an active ingredient in dentifrices (73-75).

Polyhexamethylene guanidine phosphate (PHMG-P) has been recently introduced for control of infections in dentistry. So far only limited data are available with respect to its antibacterial activity against periopathogens and its clinical efficacy in the treatment of periodontitis.

2 AIMS OF THE THESIS

2.1 Overall objective

The overall aim of the thesis was to investigate the antimicrobial, anti-inflammatory and cytotoxic properties of PHMG-P in preclinical studies and then to evaluate its efficacy under clinical conditions as an irrigant in the hygiene phase of periodontal treatment.

2.2 Specific goals

- 2.2.1** To define anti-microbial activity of PHMG-P against periopathogenic, cariogenic and standard quality control microorganisms compared to CHX, using the quantitative suspension method (Paper I).
- 2.2.2** To investigate the cytotoxicity of PHMG-P compared to CHX, and their potential modulatory effects on secretion of inflammatory mediators and matrix metalloproteinase (MMP) -1 by human gingival fibroblasts (Paper II).
- 2.2.3** To evaluate the clinical effects of intrasulcular irrigation with PHMG-P and CHX as adjunctives to periodontal debridement in patients with severe chronic periodontitis (Paper III).
- 2.2.4** To study the microflora and cytokine profiles of periodontal pockets following SRP with adjunctive antiseptic irrigation by PHMG-P or CHX (Paper IV).

3 MATERIALS AND METHODS

3.1 Ethical considerations

The study on human gingival fibroblasts, including the collection of gingival biopsies (*Paper II*), was approved by the Ethics Committee of Huddinge University Hospital, (#377/98).

The clinical trial (*Paper III*) was conducted according to the principles outlined in the Declaration of Helsinki on experimentation involving human subjects and approved by the Ethical Board resolution of Belarusian State Medical University N.5 of 18.04.2011. Every patient received written and oral information about the study, the substances to be used, and treatment methods. The participants also received a contact telephone number, in case of additional requests or any queries. Informed consent was obtained from every subject. Participation was voluntary and the subjects were free to withdraw from the study at any time. All personal data were coded and the keys were kept separately from the codes.

As a part of the treatment routine, all patients were informed about the results of examinations and diagnoses. They received no payment for their participation.

3.2 Antiseptics

The working concentrations of antiseptics applied in clinical settings correspond to those dispensed in the original commodity package. The 1% PHMG-P preparation is marketed commercially as Aquin. A range of CHX concentrations from 0.005 to 2% is available for chairside administration. In our clinical study we used 0.2% CHX solution. Thus the working concentrations of the antiseptics were 1% PHMG-P and 0.2% CHX.

In *Papers I and II*, antiseptic solutions were prepared *ex tempore* in dH₂O: PHMG-P from 70% gel, with an average molecular weight of 4000-9000 (Institute of Eco-Technological Problems, Moscow, Russia) and CHX from 1% CHX (Apotek Produktion & Laboratorier AB, Stockholm, Sweden).

In *Papers III and IV* the commercial antiseptic Aquin (Inkraslav©, Minsk, Belarus), containing 1% (w/v) PHMG-P as the active substance, and 0.2% (w/v) chlorhexidine (Public Pharmaceutical Service, Minsk, Belarus) were used as adjunctive irrigants for SRP.

3.3 Laboratory Methods

3.3.1 Bacterial strains and culture

The anti-bacterial activity of the antiseptics was tested against the following strains: *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 11229), *Pseudomonas aeruginosa* (ATCC 15412), *Candida albicans* (ATCC 1023), *Porphyromonas gingivalis* (ATCC 33277), *Aggregatibacter actinomycetemcomitans* (HK 1519), *Streptococcus mutans* (CCUG 27624; Ing-Brit) and *Lactobacillus acidophilus* (NCTC 1723). The strains of *S. aureus*, *E. coli*, *P. aeruginosa* and *C. albicans* were inoculated on trypticase soy agar (TSA) (Becton Dickinson, NJ, USA) and incubated for 18-24 hours at 37°C. The number of these microorganisms suspended in sterile PBS was adjusted by optical density (OD) and determined precisely by the drop count method. *P. gingivalis* was cultured on Colombia base agar (Acumedia, Baltimore, MD, USA) supplemented with hemin (0.05 mg/ml) (Sigma-Aldrich, Sweden AB), vitamin K (0.01 mg/ml) (BBL™, Becton Dickinson), and citrated horse blood (5%) (Sigma-Aldrich), in an anaerobic atmosphere (GasPak, Becton Dickinson) for 7 days. The test suspension of *P. gingivalis* with OD 0.73-0.75 was prepared in Peptone Yeast Glucose broth (Becton Dickinson). A two day culture of *A. actinomycetemcomitans*, grown on Colombia base agar supplemented with 0.01% tryptophan and 5% citrated horse blood was suspended in Haemophilus Teat Medium (HTM) broth (Bacto™, Becton Dickinson). The OD of the suspension was adjusted to 0.76- 0.78. *S. mutans* was cultured on Brain Heart Infusion (BHI) (Oxoid, Malmö, Sweden) agar for two days and the suspension, with an OD of 0.14 was prepared in BHI broth. *L. acidophilus* was grown on *Lactobacilli* MRS (de Man, Rogosa and Sharpe) medium (Difco™, Becton Dickinson) for two days and the test suspension with an OD of 0.14 - 0.15 was prepared in *Lactobacilli* MRS broth. The reference strains of *S. mutans*, *A. actinomycetemcomitans*, *L. acidophilus* were cultured in a capnophilic atmosphere containing 5% CO₂. The turbidity of all suspensions was standardized for each bacterial strain spectrophotometrically at 580 nm (Biochrom WPA CO7500 Colorwave Colorimeter), to provide a concentration of test microorganisms of approximately 1×10⁸ colony-forming units/ml (CFU/ml). All bacteria were passed through 5 µm filters (Pall Corporation, USA) to avoid clustering.

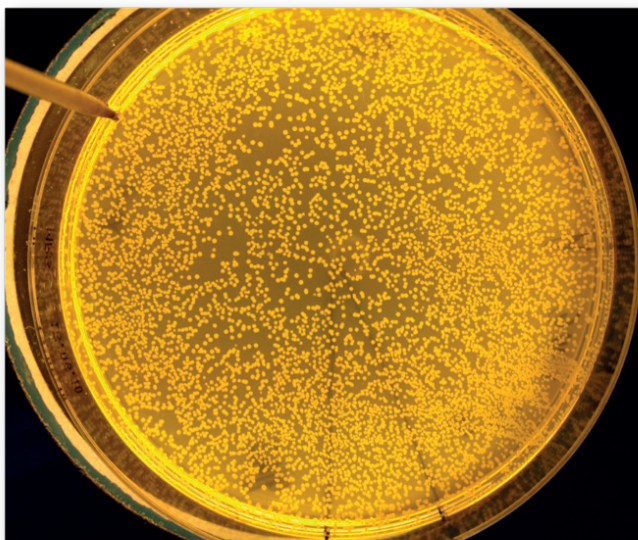


Figure 1. L. acidophilus cultured on Lactobacilli MRS medium.

3.3.2 Neutralizing solution

Neutralizing solution was added to the mixture of bacteria and antimicrobials to stop the antiseptic action. Neutralizing solution, containing 1% (w/v) peptone, 3% (w/v) Tween 80 (BDH, Poole, UK), 0.3% (w/v) lecithin (Fisher Scientific, Loughborough, UK), 0.1% (w/v) histidine (BDH) and 0.1% (w/v) cysteine (Sigma-Aldrich) was prepared in dH₂O and sterilized.

3.3.3 Evaluation of bactericidal activity

The bactericidal activity of the anti-microbials was tested by the quantitative suspension method (Figure 2). Briefly, 50 µl aliquots of the test suspensions of microorganisms with cell titer 1.0×10^8 CFU/ml were exposed to 450 µl of each antiseptic (PHMG-P or CHX), at concentrations of 0.05, 0.2, 0.5, and 1% for 30 s, 1, 3 and 5 min, followed by transfer of the mixture to 450 µl of neutralizing solution. After 10 min neutralization, serial dilution (10^{-3} , 10^{-4} , and 10^{-5}) was performed and 100 µl aliquots were plated on the appropriate media and incubated at 37°C for 2 to 7 days, depending on the bacterial species. The colonies were counted to assess the viability of the test mixture. Antiseptic activity was evaluated as the reduction factor (RF), calculated as the difference between logarithms of CFU/ml values before and after exposure to tested concentrations of the antiseptics. The sensitivity threshold of the method was estimated to be 1.0×10^3 CFU/ml. If the antiseptic application resulted in total suppression of bacteria, defined as no growth

state, cell titer was presumed to be under 1.0×10^3 CFU/ml. For each product and time of exposure, the bactericidal effect of the antiseptic agents against the test species was recorded in duplicate during two consecutive runs.

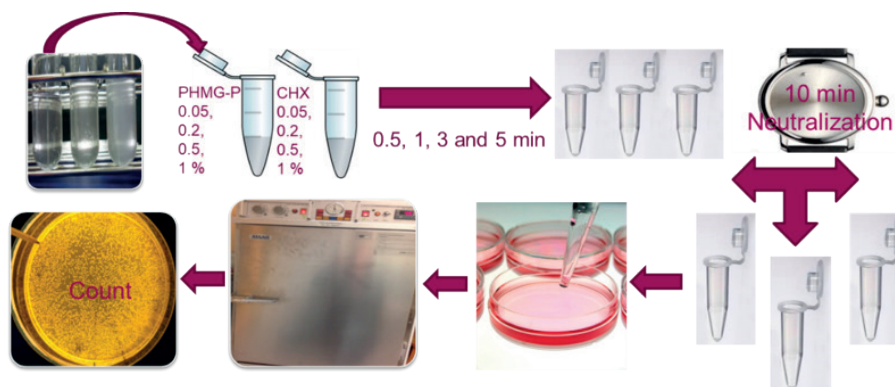


Figure 2. Flow diagram of the quantitative suspension method for testing bactericidal activity.

3.3.4 Cell culture

For *Paper II* human gingival fibroblasts (HGF) were isolated from gingival biopsies and cultured as previously described, or purchased from CLS Cell Lines Service GmbH, Eppelheim, Germany [17]. Minced gingival explants were transferred to 25 cm³ Falcon tissue culture flasks containing 5ml of DMEM (Invitrogen Life Technologies, Scotland, UK) supplemented with 4mM L-glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin and 5% foetal calf serum (Invitrogen, Scotland, UK). The fibroblasts were cultured at 37°C in a humidified incubator with 5% CO₂ and routinely passaged at 80% confluence, using 0.025% trypsin in PBS containing 0.02% EDTA (Invitrogen, Scotland, UK). Cells sampled from passages 10 to 14 were subsequently used in the studies.

3.3.5 Cell viability test

The effect of the antiseptics on fibroblast viability was estimated colorimetrically by tracing live cells in culture as described by Mosmann (76). The assay is based on the ability of living cells to take up and convert tetrazolium bromide to formazan in oxidoreductive mitochondrial processes (Fig 3).

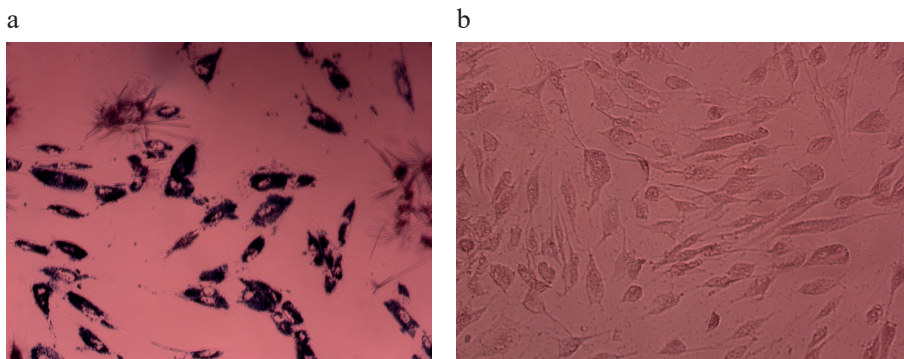


Figure 3. Human gingival fibroblasts after the first step of the cell viability test. Vital spindle-like fibroblasts are coloured blue due to the formazan pigmentation (a). Non-vital HGF were unable to uptake and process tetrazolium bromide (b), magnification x20.

HGF were seeded on 96-well tissue culture plates at a concentration of 1×10^4 cells/well and cultivated for 24h. The cells were rinsed twice with serum-free DMEM, followed by the addition of serum-free medium containing PHMG-P or CHX at concentrations of 0.00005, 0.00009, 0.0005, 0.0009, 0.005, 0.009, 0.05, 0.1, 0.2, 0.5 and 1% (w/v). The cells were incubated for another 24h prior to determination of cell viability, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in accordance with the manufacturer's instructions (Abnova, Taipei, Taiwan). Cells killed with saponin were used as a negative control. The cells were also treated with PHMG-P or CHX at 0.005, 0.05, 0.1, 0.2, 0.5 and 1% (w/v) concentrations for 1, 3, 15 and 30 min. The fibroblasts were rinsed twice with media supplemented with serum and analyzed for cell viability. The OD of the reaction product was measured by spectrophotometry (Labsystems Multiskan MS, Helsinki, Finland) at 540 nm. Cell viability was then scored according to the following formula: Cell viability (%) = (OD of the test group – OD of the positive control)/(OD of the negative control group – OD of the positive control) x 100. All cell culture experiments were carried out in quadruplicate in two independent series.

3.3.6 Stimulation of gingival fibroblasts with IL-1 β

The effects of PHMG-P and CHX on the pro-inflammatory molecules PGE2, IL-6, IL-8 and collagenase MMP-1 secreted by gingival fibroblasts were assessed in non-stimulated cells and in cells stimulated with IL-1 β . HGF were seeded at a concentration of 5×10^5 cells/well on 24-well plates. Following overnight incubation, the cells were rinsed twice with serum-free media and treated with the antiseptics CHX and PHMG-P at the respective concentrations 0.000045% and 0.00009% (w/v) separately, or in combination with IL-1 β (300pg/ml) (R&D Systems Inc., Minneapolis, USA) for 24h. Serum-free DMEM or IL-1 β served as negative and positive controls, respectively. The conditioned media supernatants were collected

and stored at -80°C for further analysis. The PGE_2 , IL-6, IL-8 and MMP-1 levels in the supernatants were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits for PGE_2 (Cayman Chemical, Ann Arbor, USA), IL-6, IL-8 and MMP-1 (R&D Systems Inc., Minneapolis, USA) according to the manufacturer's instructions. Absorbance values were recorded at 450 nm by a microplate spectrophotometer (Labsystem Multiskan MS) with wavelength correction at 540 nm. Each antiseptic treatment was performed and analyzed in triplicate.

3.3.7 Biomarker Immunoassays

The levels of IL-1 β , IL-8, IL-10 and IL-17A were determined using multiplex immunoassay, according to the manufacturer's instructions (ProcartaPlexTM Multiplex Immunoassay, ThermoFisher, Vienna, Austria). The readings were recorded using Bio-Plex 100 (Bio-Rad Laboratories, Inc., CA, USA). The assay ranges were as follows: for IL-1 β : 0.32-1330 pg/mL; for IL-10: 0.18-750 pg/ml and for IL-17A: 0.25-1030 pg/ml.

MMP-8 levels were measured by time-resolved immunofluorimetric assay (IFMA) as described earlier (77, 78). Briefly, the MMP-8 specific monoclonal antibodies 8708 and 8706 (Medix Biochemica, Kauniainen, Finland) were applied as a capture antibody and a tracer antibody. The tracer antibody was labelled with europium-chelate and the assay buffer contained 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 5 mM CaCl_2 , 50 μM nCl_2 , 0.5% bovine serum albumin, 0.05% sodium azide and 20 mg/l diethylenetriaminepentaacetic acid. GCF samples were diluted in assay buffer and incubated for 1 h, followed by another 1 h of incubation with tracer antibody. Enhancement solution was added and incubation fluorescence was measured after 5 min, using a 1234 Delfia Research Fluorimeter (Wallac, Turku, Finland). The detection limit for this assay was 0.08 ng/ml.

Due to the heterogeneity of the samples i.e. variation in human material because of manual sampling, GCF samples were normalized to a total protein concentration. The total concentration of proteins was measured in each sample using a fluorescence-based assay according to the manufacturer's instructions (Qubit Fluorimeter, Life Technologies, USA). Immune marker concentrations were adjusted to total protein concentration and expressed as a ratio (pg of immune marker/mg of total protein).

3.3.8 DNA extraction and quality control

DNA was extracted from clinical samples using a "Nucleosorb-C" kit (Primetech, Minsk, Belarus) according to the manufacturer's instructions. DNA quality and concentration were estimated by spectrophotometry at wavelengths of 260/280 nm, 260/230 nm, 260/320 nm (SOLAR PB2201, Minsk, Belarus). The majority

of samples reached values of $260/280 = 1.7-1.9$ and molar concentration $>5 \text{ ng}/\mu\text{l}$. Finally, some DNA samples were analyzed with 1% agarose gel electrophoresis to evaluate the degree of fragmentation (formaldehyde was not added to avoid denaturing).

3.3.9 Primers and probes for real time PCR

Oligonucleotide sets, including probes developed and validated by other researchers, were used in this study (Table 1) (79-82). To optimize the reaction and increase its efficiency, some sequences were adjusted (Table 1). They were partially modified with locked nucleic acid (LNA) nucleotides. In each case, we selected the ratio of oligonucleotides in the reaction mixture, and optimized the buffer conditions to predict and avoid formation of secondary structures revealed by “The mfold Web Server” and “Oligoanalyzer” (Integrated DNA Technologies, Inc., USA) (83, 84). The oligonucleotide sequences and modifications are shown in Table 1.

Table 1. Oligonucleotide sequences used in related research and our modifications.

Species	Sequences	Our modifications
<i>P. gingivalis</i> (81)	Forward TGGTTTCATGCAGCTTCTTT Reverse TCGGCACCTTCGTAATTCTT Probe FAM- CGTACCTCATATCCCGAGGGGCTG-BHQ1	- - -
<i>T. denticola</i> (81)	Forward CCTTGAACAAAAACCGGAAA Reverse GGGAAAAGCAGGAAGCATAA Probe FAM- GAGCTCTGAATAATTTTGATGCA-BHQ1	- - -
<i>F. nucleatum</i> (80)	Forward GGATTTATTGGGCGTAAAGC Reverse GGCATTCTACAAATATCTACGAA Probe FAM-CTCTACACTTGTAGTTCGG	GG[LNA-A] TTTATTGGGCGTAAAGC -
		CTCTACACTTGTAGTT[LNA-C] [LNA-C]G
<i>T. forsythia</i> (82)	Forward GAGGTTGTGGAAGGTATG Reverse GTAGATCAGAATGTACGGATT Probe FAM-TCTCCGCTTATTCGTGAC-BHQ1	- - -
<i>A. actinomyce- temcomitans</i> (82)	Forward GCGAAACGAAGAGAAGCAAG Reverse CCTACCCAACAGGCGTATCA Probe FAM-ATTCCCAACCGCACTT-BHQ1	- - FAM-ATTCCCAAC[LNA-C] GCACTT-BHQ1
<i>P. intermedia</i> (79)	Forward TGTCGGTTTACTGGCTATGTTCTC Reverse CTTGTCTGTTGGCCATCTTGAAG Probe FAM- TCAAAGACGCACGTACCAATCCAGACC- BHQ1	- - -

3.3.10 Real time PCR

Real time PCR was conducted by CFX96touch (Bio-Rad, USA) according to the following protocol. The reaction mix (25 µl volume contained: 500 nM of each oligonucleotide, including the probe, 2 mM MgCl₂, 0.1 mM of each dNTP, 1x PCR commercial buffer without MgCl₂, 1.25 U of thermostable Taq DNA-polymerase without hot start, deionized RNase/DNase free water for PCR. The cycling conditions were: +95°C for 3 min – primary denaturation, then 50 cycles: +95°C for 10 s and +60°C for 59 s (annealing and elongation steps).

3.3.11 Standard curves and reaction efficiency

To plot standard curves for microbes the mixture of 10 different DNA samples was used with the highest concentrations resulting from DNA purification. Before DNA purification, each sample was collected from a separate patient. This approach is intended to minimize the effect of the sample type and the isolation method on the reaction efficiency.

Quantitation cycle (C_q) values were determined in DNA samples and those with C_q < 20 were selected. The DNA was then pooled and mixed in one tube, vortexed and serially diluted 5-fold. This yielded 5 calibrators with 5-fold steps of DNA concentration. The data on reaction efficiency and R² parameters are presented in Table 2. In all cases the recorded R² values exceeded 0.99, which is consistent with the data reported in the literature. At the same time, in most of our experiments the recorded efficiency of the reactions was superior to that of previous studies.

Table 2. Comparison of reaction efficiency and R² values of the present study with the previous literature data.

Target species	Eff. (current)	Eff. (previous)	R ² (current)	R ² (previous)
<i>P. gingivalis</i>	96.5%	91.0% (81)	0.997	0.999 (81)
<i>T. denticola</i>	99.3%	7.0% (81)	0.998	1.000 (81)
<i>F. nucleatum</i>	99.7%	90-100% (85)	0.996	0.997 (85)
<i>T. forsythia</i>	97.0%	88.0% (82)	0.998	1.000 (82)
<i>A. actinomycetemcomitans</i>	93.5%	73.7% (82)	0.999	0.999 (82)
<i>P. intermedia</i>	95.6%	121.2% (79)	0.994	0.995 (79)
SCARNA5	97.8%	-	0.998	-

3.3.12 PCR data analysis

Heterogenic microbial DNA samples were normalized by human SCARNA5 (ID: 677775) NC_000002.12 gene. The gene possesses a DNA sequence which is not replicated in multiple copies in the human genome and does not contain specific

sites for microbial oligonucleotide annealing. The oligonucleotide sequence was designed on primer 3 v. 0.4.0 and a subsequent analysis with mfold and IDT Oligoanalyzer (86-88). The resulting oligonucleotide sequences are Forward CCTCCCGTCACATTTAAGTCA; Reverse GCCGATCACTCTCAGAAACAC; Probe FAM-TCATGGAGCAGCTGATAATTTG-BHQ1.

Using a cell culture of human keratinocytes, we made approximate counts of human SCARNA5 gene copies for each amplification cycle during standard curve plotting, using only human DNA with known concentrations. The reaction efficiency differed slightly between the clinical samples and the cell culture (97.8% and 98.9% respectively) as well as R^2 0.998 and 0.992, respectively.

Cq equal to 25 of 21.4 ng/μl corresponded to 35780 copies per reaction in the run graph. The difference of 1.2% in reaction efficiency was negligible.

The number of SCARNA5 copies in each clinical sample was calculated using quantitative calibration. The number of copies of the microbial genome-equivalents was then computed for each target, based on corresponding copies at the start of each amplification cycle. The obtained data were used for further statistical analysis.

Real time PCR data were analyzed using BioRad CFX96touch basic software. The obtained Cq data were converted to MS Excel format for subsequent analysis.

Due to gentle collection technique the samples contained insufficient amount of human material for normalization. The absolute number of microbial gene equivalents is further presented, analyzed and discussed.

3.4 Clinical study

3.4.1 Clinical study design

The study was a single center, double-blind randomized prospective clinical trial. To avoid selection bias, a simple randomization technique based on the throw of a dice was used. All patients were allocated to one of 3 groups according to the score on the dice cast by the nurse: scores 1 or 4 went to the first group, 2 or 5 to the second group and scores 3 or 6 to the third group. In the first group (19 subjects) Aquin (Inkraslav©, PHMG-P 1%), was applied as an adjunctive irrigant for SRP; in the second group (21 subjects) the irrigant used was 0.2% CHX (Public Pharmaceutical Service, Minsk, Belarus), and the third group (19 subjects) served as the control, with distilled water irrigation.

3.4.2 Patient enrolment, examination and treatment

The participants were recruited into the study between autumn 2011 and spring 2014 from patients referred to the Periodontal Clinic of the School of Dentistry, Belarusian State Medical University. The inclusion criteria stipulated that the subjects should be aged 18 to 75 years, possess normal health status, with diagnosed generalized or local severe chronic periodontitis, have at least 3 teeth with periodontal pockets of minimum probing depth of 6 mm, and radiographic evidence of extensive bone loss (\geq one third of root length). In compliance with a new classification system for periodontal and peri-implant diseases and conditions, such a condition is referred to as chronic periodontitis stage III or IV grade B (1). The Armitage 1999 diagnostic keys and definitions were used as they were widely applied at the time of the study (6). The reasons for exclusion from this study, apart from failing to meet the aforementioned criteria, were as follows: periodontal treatment performed less than six months prior to the study, pregnancy, nursing mother, symptoms which might affect progression of periodontal disease or which require administration of antibiotics, allergy to CHX, PHMG-P, or a course of antibiotics in the previous 6 months.

At baseline the patients underwent a comprehensive periodontal examination. Panoramic radiographs were used to verify the diagnosis of chronic severe periodontitis. The presence of dental plaque at the gingival margin along the mesial, buccal, distal and lingual aspects was determined, and the plaque index (PI) was calculated. Gingival inflammation was registered as bleeding on probing (BOP) and expressed as the proportion of bleeding sites relative to the total number of sites. Pocket probing depth (PPD), regarded as the primary outcome, was measured to the nearest mm using a calibrated Williams' periodontal probe (Falcon®, Sialkot, Pakistan). PPD was defined as the distance between the gingival margin and the deepest point of probe tip penetration into the periodontal pocket at six sites measured on each tooth. Periodontal pockets with PPD > 4 mm were considered to be pathological and were selected for analysis (89). PPD of 6 mm was chosen as the threshold value for surgical treatment need. After the baseline examination, all subjects received initial periodontal therapy, which included motivation, oral hygiene instruction and full-mouth debridement, using a combination of ultrasonic and manual instrumentation, with one of the test solutions as an irrigant. The irrigants were delivered to the periodontal pockets at a dose of 2 ml per periodontal pocket, by means of a syringe with a blunt needle. At each follow-up appointment, all treatment procedures were repeated. After baseline, five follow-ups were scheduled: at 2 weeks and 1 month, and then at 4, 6 and 12 months.

Full mouth debridement with adjunctive irrigation took up to two hours. Scaling was performed with the help of universal curettes, by adjusting 2 mm of the tip and stroking twice per each mm of the root. Over the period of the study subsequent appointments for debridement were less time-consuming, as the scaling

need decreased. Local anesthetics were used if necessary, e.g. Ubistesin® (3M ESPE, Seefeld, Germany). All irrigant solutions were prepared by a nurse, who randomly chose the code and distributed the solutions in identical opaque bottles with corresponding numbers. The legend was sealed until statistical analysis.

3.4.3 Collection of GCF and bacterial samples

For *Paper IV* gingival crevicular fluid (GCF) and bacterial samples were collected from patients participating in the clinical trial (*Paper III*) at baseline, at 2 weeks, 1 and 4 months.

In order to avoid bleeding GCF samples were taken before clinical examination and bacterial sampling. Each tooth was isolated with cotton rolls and air dried. The contents of the periodontal pockets were extracted by paper strips PerioPapers® (Oralflow Inc., NY, USA) inserted for 30 s into 4 periodontal pockets, one from each quadrant. The same periodontal pockets were used for sampling at each follow-up. Strips contaminated with blood were discarded. The strips were placed separately in empty test tubes, immediately frozen at -20°C, transferred to a freezer within a week and maintained at -80°C until further processing.

Bacteria were sampled by scaling from the deepest 4 periodontal pockets, each representing a quadrant of the dentition. In order to harvest similar-sized samples from each pocket, 2 strokes of the scaler were made in each pocket. The content was then pooled in a test tube filled with sterile transport medium and frozen at -20°C until DNA extraction.

3.5 Statistical analysis

The data from patients (*Paper III*) were analyzed as they were randomized. If the patient withdrew from the study the data were stored and analyzed for the actual period. Descriptive statistical data were computed and expressed as the mean \pm standard deviation (SD). The Friedman Test was applied to reveal differences within treatment groups and the Kruskal-Wallis Test was applied for intergroup comparisons. To estimate the significance of differences between two groups, the Mann-Whitney Test was applied to independent groups and Wilcoxon Signed Rank Test to dependent groups. To evaluate the interrelationship of the observed parameters Spearman's rank correlation coefficient was chosen. P-value = 0.05 was defined as statistically significant. In order to achieve 80% power in detecting an average inter-group PPD difference of 1 mm (assumed SD \pm 1.5 mm) the minimum sample size was estimated to be 14 patients in each group. Oversampling was planned to allow for possible withdrawals and data corruption.

Data processing was conducted using the software packages GraphPad Prism 6 (GraphPad Software, San Diego, California USA) for *Paper II* and SPSS Statistics 25 (IBM®, SPSS© Statistics, NY, USA) for *Papers III* and *IV*.

4 RESULTS AND DISCUSSION

4.1 Activity of clinically relevant working concentrations of CHX and PHMG-P against standard quality control microorganisms

Mature biofilm exhibits enhanced tolerance for antimicrobial agents and the initial proposal was to determine antimicrobial activity on biofilm, rather than on planktonic bacteria (15). However, to date there is a lack of standardized methods for such assays on biofilm. Moreover, biofilm properties differ, depending on the surrounding environment. For example, *Acinetobacter baumannii* shows different resistance profiles in laboratory media and on *ex vivo* human ascites (90). In the present study the bactericidal effects of PHMG-P and CHX were tested on cultures of standard bacterial species, periodontopathogenic and cariogenic bacteria, using the quantitative suspension method. The results are summarized in Figures 4-7.

Clinically relevant working concentrations of PHMG-P (1%) and CHX (0.2%) exhibited similar patterns of antimicrobial activity against standard quality control microorganisms (*S. aureus*, *E. coli*, *P. aeruginosa* and *C. albicans*) (Fig 4). 1% PHMG-P and 0.2% CHX exerted rapid bactericidal effects on all species within 30 s of exposure and reduced the bacterial titer to below the detection limit (RF over 5).

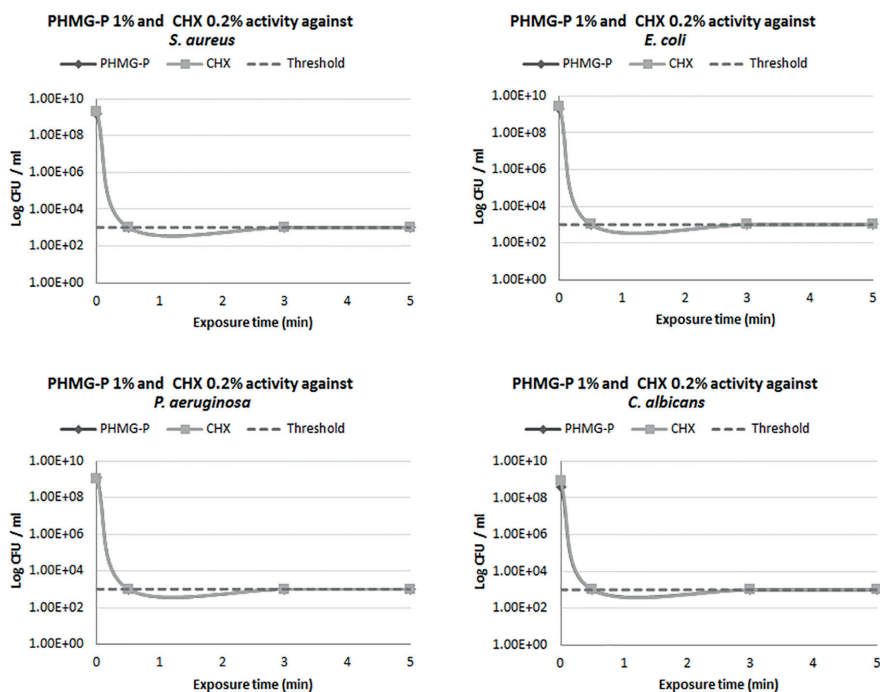


Figure 4. Antimicrobial activity of clinically relevant working concentrations of PHMG-P (1%) and CHX (0.2%) against standard quality control microorganisms (*S. aureus*, *E. coli*, *P. aeruginosa* and *C. albicans*).

4.2 Activity of clinically relevant working concentrations of CHX and PHMG-P against periopathogens and cariogenic microorganisms

Both tested antiseptics PHMG-P and CHX at working concentrations 1 and 0.2%, respectively, exerted a rapid bactericidal effect (within 30 s) against *A. actinomycetemcomitans* and *P. gingivalis* (Fig 5). In contrast to the periopathogens, stricter PHMG-P and CHX application modes were required to control cariogenic bacteria. Even after 5 min of treatment, neither 1% PHMG-P nor 0.2% CHX were able to eliminate *L. acidophilus* (Fig 5). Exposure of thirty seconds to 1% PHMG-P or 0.2% CHX failed to achieve a significant reduction in the *S. mutans* count, with RF values equaling 2.3 and 3.88, respectively. (Fig 5). Following prolonged treatment, up to 3 min, working concentrations of the antiseptics completely inhibited *S. mutans* (RF 5.8 and 5.73, respectively).

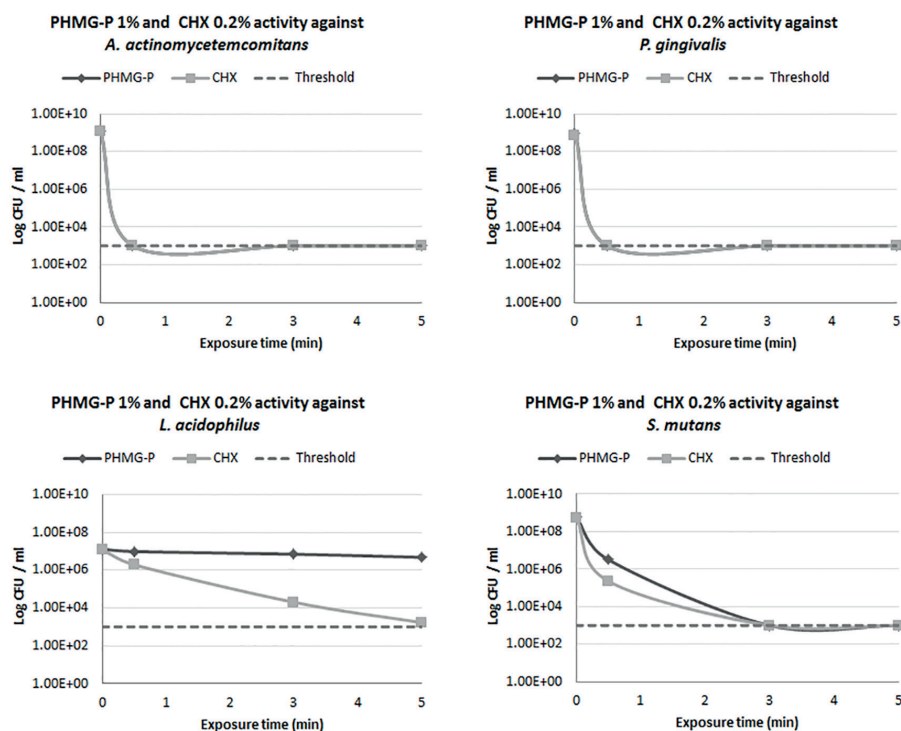


Figure 5. Antimicrobial activity of clinically relevant working concentrations of PHMG-P (1%) and CHX (0.2%) against periopathogens and cariogenic microorganisms (*P. gingivalis*, *A. actinomycetemcomitans*, *S. mutans* and *L. acidophilus*).

4.3 Activity of diluted CHX and PHMG-P against standard quality control microorganisms

Once introduced into the oral cavity, antiseptics are gradually diluted in saliva and their activity tends to decline over time (91). Thereafter, antiseptics, even at low concentrations, display protracted effects until inactivation. Therefore, it appears logical that antimicrobial activity should be evaluated not only in the range of clinical working concentrations of antiseptics but also at several dilutions and different periods of exposure. The bactericidal effects of antiseptics should be assessed for 4-20 fold dilutions and a relevant time span of 30 s to 1-5 min.

Diluted to 0.05% concentration, neither antiseptic was able to eliminate *S. aureus*, *E. coli*, *P. aeruginosa* and *C. albicans* after exposure of 30 s (Fig. 6). Prolonging treatment time for 3 min resulted in a strong bactericidal effect against *S. aureus* and *E. coli*, accompanied by a pronounced fall in microbial titer, below the sensitivity threshold of the method. This study demonstrated that with prolonged exposure, antiseptics even at dilutions of 4-20-fold, exerted antimicrobial activity against oral microorganisms *in vitro*. Exposure to 0.05% PHMG-P totally eliminated *P. aeruginosa* and *C. albicans* within 3 min, whereas 0.05% CHX produced the same effect within 5 min (Fig 6).

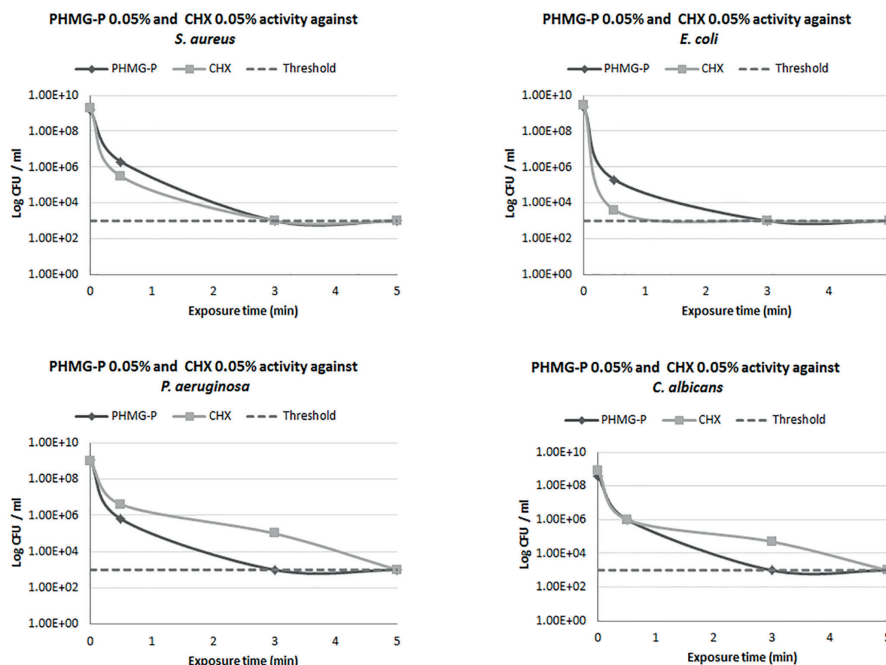


Figure 6. Antimicrobial activity of highly diluted PHMG-P (0.05%) and CHX (0.05%) against standard quality control microorganisms (*S. aureus*, *E. coli*, *P. aeruginosa* and *C. albicans*).

4.4 Activity of diluted PHMG-P and CHX against periodontopathogenic and cariogenic bacteria

Highly diluted PHMG-P (0.05%) and CHX (0.05%) acting for 30 s exhibited milder impact on *A. actinomycetemcomitans* and *P. gingivalis*, characterized by RF values under 4 (Fig 7). For both antiseptics, prolonging exposure to 3 min resulted in total elimination of the bacteria (RF ≥ 5.86).

L. acidophilus culture was not critically affected by the antiseptics at 0.05% concentrations after 5 min exposure. PHMG-P and CHX caused only a minor drop in microbial titers, with RF values 0.24 and 0.51, respectively (Fig 7).

The limited antimicrobial impact on *S. mutans* following 30 s exposure to 0.05% PHMG-P or CHX action is reflected in the respective RF values - 0.28 and 0.99 (Fig 7). Longer exposure (3 min) to 0.05% PHMG-P and 0.05% CHX also demonstrated insufficient antimicrobial activity (RF 3.55 and 3.73, respectively). Prolonged exposure (5 min) diminished *S. mutans* titer below the sensitivity threshold of the method.

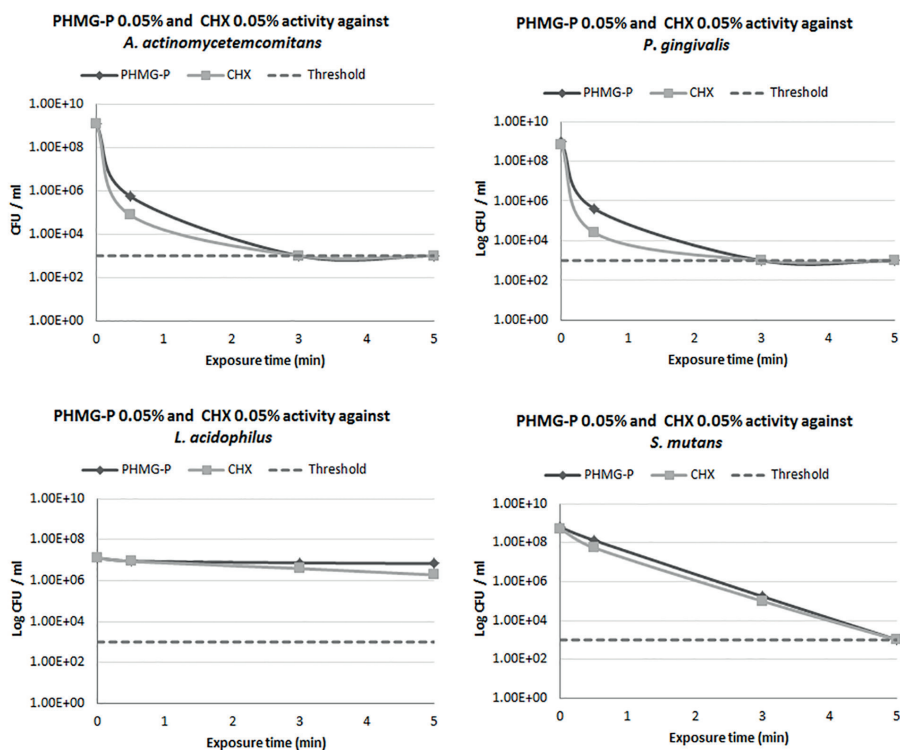


Figure 7. Antimicrobial activity of highly diluted PHMG-P (0.05%) and CHX (0.05%) against periodontopathogens and cariogenic bacteria (*P. gingivalis*, *A. actinomycetemcomitans*, *S. mutans* and *L. acidophilus*)

Strong bactericidal activity of 1% PHMG-P expressed within 30 s against *P. gingivalis* and *A. actinomycetemcomitans* and the activity retained after 20-fold dilution and exposure of 3-5 min suggests that PHMG-P may be a promising antiseptic for periodontal disease management and prophylaxis. According to our data, PHMG-P at a concentration of 0.05% required 3 min to achieve bactericidal effects against the majority of bacteria. Owing to the potent biocidal activity expressed within a short time against *S. mutans*, *S. aureus*, *C. albicans*, as well as toward other quality control microorganisms and potential dental plaque constituents, PHMG-P warrants evaluation for application in caries prevention.

4.5 Effect of PHMG-P and CHX on viability of human gingival fibroblasts

When assessing prospects of antiseptic agents for clinical application, not only antimicrobial activity but also possible cytotoxicity to human tissues must be taken into account. The effects of antiseptics on human gingival fibroblasts were investigated in terms of the number of vital cells remaining after exposure to the antiseptic for either 24 h or 1-30 min.

Initially we determined the maximum non-toxic concentration of the antiseptics that is, the concentration above which exposure of 1 min to the antiseptic could be lethal to cells. The maximum concentration of antiseptics not fatal to HGF was 0.005% (Fig 8A). This value is in fact lower than those applied in clinical medicine (0.05-1%) and those exhibiting antimicrobial activity in our previous study (0.05%) (92). However, *in vitro* gingival fibroblast culture does not faithfully replicate the environment in the human oral cavity, where protection is provided, not only by epithelial cells but also by saliva, which gradually dilutes the concentration of the antiseptics (93). This concentration was selected for testing during a prolonged exposure period of 1 to 30 min. Five min of contact with 0.005% PHMG-P caused loss of cellular vitality, whereas cells survived even after 30 min exposure to 0.005% CHX (Fig 8B).

Long-term exposure (24 h) of cells to PHMG-P at concentrations higher than 0.00009% resulted in loss of fibroblast vitality. In contrast, inhibition of fibroblasts by CHX occurred at concentrations higher than 0.0009% (Fig 8C). PHMG-P severely disrupted fibroblast survival during 24 h exposure at concentrations ten times lower than CHX, demonstrating a stronger cytotoxic effect than the gold standard, CHX. The greater cytotoxic potential of PHMG-P compared with CHX may be attributed to its polymeric structure, which enhances adherence of the antiseptic to the tissue surface (94, 95).

An outbreak of fatal idiopathic lung disease has been reported in South Korea (96). The suspected cause was chronic inhalation of a humidifier disinfectant (96). The disinfectant mixture included PHMG-P, poly (oxyalkylene guanidine) hydrochloride (PHMG-H), and 5-chloro-2-methylisothiazol-3(2H)-one/2-methylisothiazol-3(2H)-one (97). Extension of the application scope from disinfectants to humidifier water treatment changed the route of administration (98). Modified manner of exposure might alter the health effects of substances. In an animal model, chronic exposure to lower concentrations of PHMG-P aerosol particles (1.5 mg/m^3) might be responsible for pulmonary inflammation and fibrosis, while alimentary delivery of PHMG-P at a higher concentration (40 mg/kg/day) showed no-observed-adverse-effect levels (NOALs) (98, 99). PHMB is not absorbed when applied topically (100). One study questioned the role of PHMG-P as the cause of lung disease in Korean residents. However, this investigation could be biased, since it was financed by the manufacturer of humidifier (101). Unfortunately, after an interval of several years, new cases of clinically similar lung pathologies have been reported in South Korea. The putative causative agents are two new substances used as humidifier disinfectants: (5-chloro-2-methyl-4-isothiazolin-3-one (CMIT) and/or 2-methyl-4-isothiazolin-3-one (MIT)) (102).

Multidisciplinary experts considering the results of genotoxicity and animal studies concluded that carcinogenic risk from application of PHMB for wound antisepsis can be ruled out (100). An expert meeting in 2008 recommended polyhexamethylene biguanide (PHMB) as a highly appropriate antiseptic agent for treatment of infected wounds (103). Moreover, on the basis of clinical evidence, experts supported the use of PHMB as an antiseptic for wound treatment (100). It was mentioned earlier, that local application of PHMG-H as a mouthrinse showed good clinical results (74).

In the EU, a similar humidifier disinfectant is marketed commercially under the brand Akacid Plus® (104). In *in vitro* studies Akacid plus demonstrated a broad spectrum of antimicrobial properties and *in vivo*, animal models showed low oral and dermal toxicity (105). Its nebulization efficiently removed pathogenic nosocomial microorganisms (106). To the best of our knowledge there are no reports of idiopathic lung diseases associated with Akacid Plus. Topical application of 0.5% Akacid Plus cream was efficient in the treatment of methicillin-resistant *Staphylococcus aureus* superficial skin infection in a guinea pig model (107). It has been also shown that Akacid medical formulation induced apoptosis in leukemic cells *in vitro* and inhibited tumor growth *in vivo* (108).

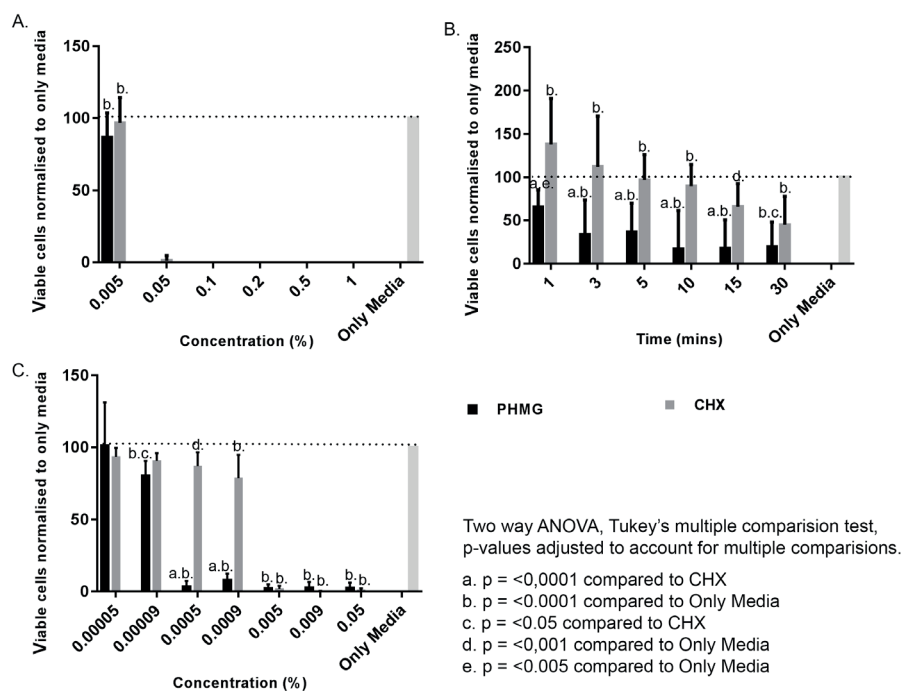


Figure 8. The effect of PHMG-P and CHX on viability of human gingival fibroblasts. *A.* Viability of human gingival fibroblasts after 1 min exposure to PHMG-P and CHX in the concentration range 0.005-1%. *B.* Dynamics of gingival fibroblast viability after 30 min treatment with either 0.005% PHMG-P or 0.005% CHX. *C.* Survival rate of human gingival fibroblasts after 24 h exposure to 0.00005-0.05% of PHMG-P and CHX.

4.6 Modulatory effect of PHMG-P and CHX on the secretion of inflammatory mediators and MMP-1 by human gingival fibroblasts

Human gingival fibroblasts were stimulated with IL-1 β to induce an inflammatory response. The effects of antiseptic administration (PHMG-P and CHX) were then monitored, by assessing the secretion of PGE₂, IL-6, IL-8 and MMP-1. PHMG-P or CHX alone did not affect the levels of PGE₂, IL-6, IL-8 or MMP-1 whereas, as expected, stimulation with IL-1 β resulted in considerably increased production of all four mediators, compared with untreated cells ($p < 0.0001$) (Fig 9A-C). The addition of PHMG-P or CHX along with IL-1 β significantly reduced the levels of PGE₂ ($p < 0.001$), as well as IL-6, IL-8 and MMP-1 synthesis by fibroblasts ($p < 0.05$) at all the concentrations tested (Fig. 9A-D).

Since the experiments assessing anti-inflammatory properties of antiseptics require overnight incubation, it is necessary to use concentrations which are low enough for the cells to withstand exposure for 24 h. In the experiment only low concentrations of antiseptics expressed anti-inflammatory properties: if high concentrations are used, the cells might perish before the anti-inflammatory properties can be expressed. On the other hand, the higher concentrations are applied, the more powerful is the resultant anti-inflammatory effect. In case of chaotropic molecules, the more molecules are available, the more molecules of pro-inflammatory mediators will be bound, resulting in a more pronounced anti-inflammatory effect. This might also be true when antiseptics bound to IL-1 β in the treatment cocktail partially neutralized the cytokine and, consequently, inflammation in cells was promoted to a lower extent compared with IL-1 β only. This aspect could be verified in future experiments by adjusting the study design and using separate sequential treatment. However, even in this case antiseptics could decrease concentration of cytokines in periodontal pockets.

The present study showed that at low concentrations PHMG-P and CHX exhibited immunomodulatory activity. Röhner et al. have previously reported the ability of PHMB but not CHX to induce morphological cell damage after a short exposure time, resulting in the secretion and accumulation of pro-inflammatory cytokines and chemokines, such as IL-1 β , IL-6, IL-7 and TNF- α (109). Conversely, in the present study neither PHMG-P nor CHX provoked an inflammatory response by the gingival cells. Moreover, treatment of IL-1 β -stimulated human gingival fibroblasts with non-toxic concentrations of both PHMG-P and CHX led to statistically significant reductions of PGE₂, IL-6, IL-8 and MMP-1 levels. Some antibiotics have previously been implicated in downregulating the expression of pro-inflammatory cytokines IL-6, IL-8 and MMP-1 by fibroblasts (110).

Inactivation of early phase inflammatory cytokines by CHX or PHMG-P may arrest the progression of periodontal diseases. However, Türkoğlu et al. failed to confirm a correlation between clinical improvement and diminished production of cytokines in GCF after CHX treatment (111).

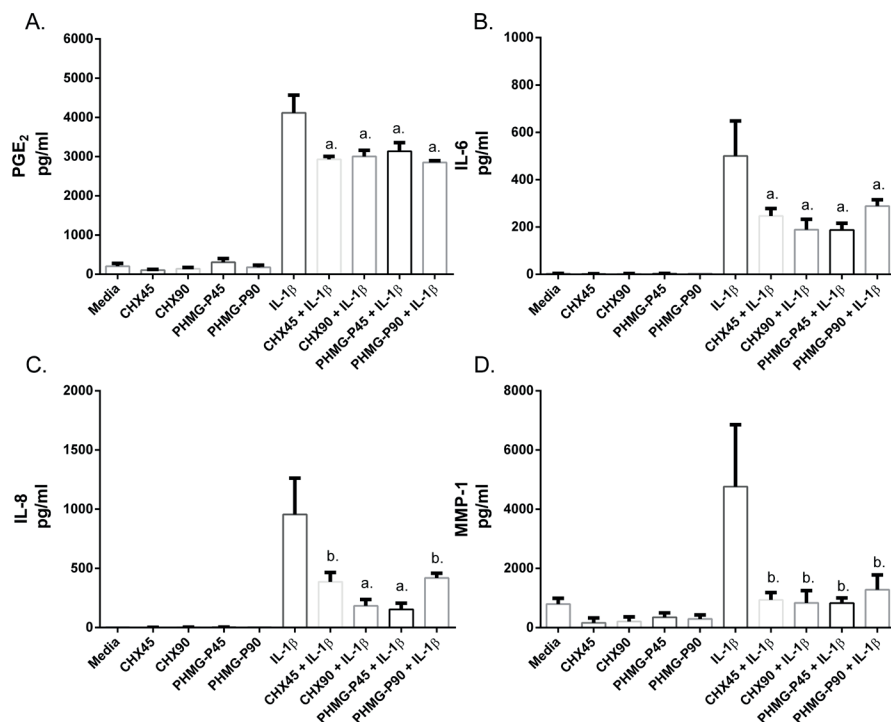


Figure 9. The effect of PHMG-P and CHX on the secretion of inflammatory mediators by human gingival fibroblasts. The levels of PGE₂ (A), IL-6 (B), IL-8 (C) and MMP-1 (D) secreted by non-stimulated or IL-1 β -stimulated human gingival fibroblasts after treatment with PHMG-P or CHX (0.000045% and 0.00009%, respectively) were assessed in cell supernatants by ELISA. Media – media only, CHX45 – CHX 0.000045%, CHX90 – CHX 0.00009%, PHMG-P45 – PHMG-P 0.000045 % and PHMG-P90 – PHMG-P 0.00009%.

4.7 Clinical study results

Fifty-nine patients (30 males and 29 females) with severe chronic periodontitis, aged 29 to 70 years, mean age 47.2 (SD 11.2) years were enrolled in the study. The demographic data are presented in Table. 4. Fifty-three patients completed the study; six withdrew, resulting in an overall retention rate of 89.8 %. The details are summarized in the flow diagram (Fig 10).

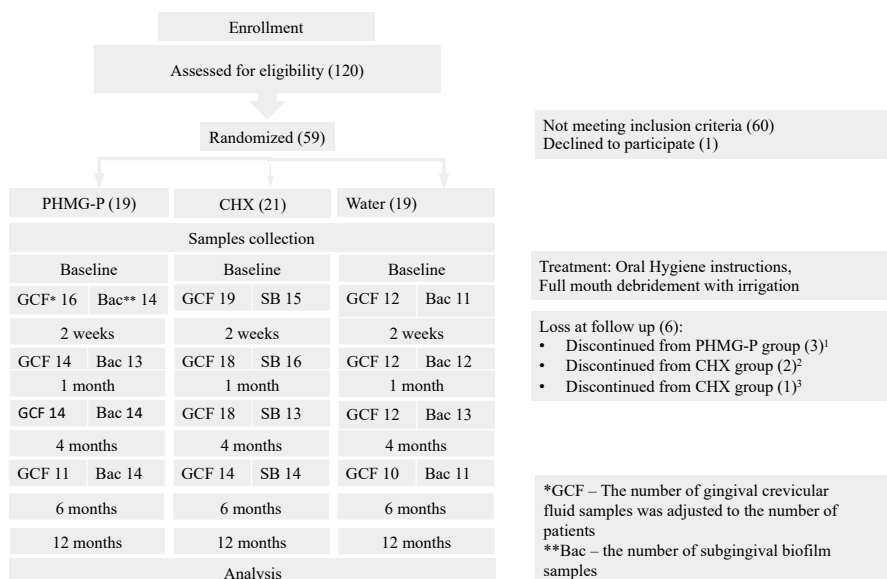


Figure 10. Flow Diagram.

Table 4. Demographic data

Variables		PHMG-P	CHX	Water	Total
Enrolled volunteers number		19	21	19	59
Number of patients completing the study		16	19	18	53
Average age (SD)		46.9±11.4	49.4±12.3	45.4±9.8	47.3±11.2
Male		10	9	11	30
Female		9	12	8	29
Smoking addicts		1	2	6	9
The number of periodontal pockets measured initially	≥ 4 mm	359	444	422	1225
	≥ 6 mm	245	250	243	738
The number of periodontal pockets around anterior teeth		118 (32.9%)	209 (47.1%)	187 (44.3%)	514

At baseline, there were no intergroup differences in PI and BOP ($p>0.05$), hence the groups were comparable (Table 5). In the course of the study PI and BOP values improved significantly within all three groups ($p<0.05$), indicating that oral debridement and hygiene caused similar effects in all the test subjects. No intergroup differences in PI and BOP were observed at any follow-up examination,

implying a weak antiseptic effect on PI and BOP. This might be due to brief and sporadic exposure to the antiseptics during debridement, possibly resulting in minor improvement, below the clinically detectable threshold. Moreover, antiseptic solutions could be rapidly washed out of periodontal pockets by gingival crevicular fluid (112). Another plausible explanation is that pathophysiological mechanisms governing the parameters PI and BOP are not sensitive to the impact of antiseptic solutions.

Table 5. Oral hygiene and bleeding on probing before and after treatment.

Treatment Group	PI, % (Mean±SD)					BOP, % (Mean±SD)				
	Baseline	1 month	4 months	6 months	12 months	Baseline	1 month	4 months	6 months	12 months
PHMG-P	35±24	20±18	26±17	22±18	16±13	33±22	15±16	16±15	12±09	7±7
CHX	33±26	16±13	15±12	15±15	11±11	31±25	15±13	13±09	12±13	7±8
Water	39±24	20±15	23±18	19±14	17±14	33±19	14±11	13±10	12±11	6±4

Periodontal pockets deeper than 4 mm were considered to be pathological and selected for PPD analysis. The results are presented in Table 6. At baseline, there were no intergroup differences. During the study, PPD decreased significantly in all three groups ($p<0.05$). Intergroup analysis revealed marked differences at one, four and six month follow-ups ($p<0.05$). Pairwise comparison disclosed significant divergence in favor of PHMG-P. No clear differences were detected by the end of the study (Table 6). The lack of intergroup differences at 12 months may be attributed to the infrequent chairside administration of the antiseptics and to the fact that in periodontal treatment adequate plaque control is a greater determinant of success than antimicrobial supplements.

Table 6. Pocket Probing Depths and reduction in periodontal pockets over 4 mm prior to and after treatment.

Treatment Group	PPD, mm (Mean±SD)					PPD reduction, mm (Mean±SD)				
	baseline	1 month	4 months	6 months	12 months	1 month	4 months	6 months	12 months	Total (12 months-baseline)
PHMG-P	6.5±1.5	5.5±1.6	5.2±1.5	5.0±1.5	5.0±1.6	-1.1±1.3	-0.3±1	-0.1±1	0.02±0.9	-1.5±1.4
CHX	6.4±1.7	5.8±1.7	5.6±1.8	5.4±1.9	5.1±1.9	-0.7±1.2	-0.2±1.4	-0.3±1.1	-0.2±1.2	-1.4±1.7
Water	6.4±1.7	5.8±1.7	5.5±1.8	5.3±1.7	5.1±1.9	-0.6±1.2	-0.3±1.4	-0.2±1.3	-0.2±1.2	-1.3±1.5

A PPD value of 6 mm was chosen as the threshold for surgical treatment need. At baseline, the mean PPD value of deep periodontal pockets (≥ 6 mm) did not differ between the groups ($p>0.05$). Significant intergroup differences in PPD, in favor of PHMG-P treatment, were detected at the one, four and six month follow-ups. However, by the end of the study, no differences were detected (Table 7).

In general, the mean PPD value was reduced by 1.3-1.5 mm. The average PPD decreased from 6.5 to 5.0 mm, which is consistent with the results of other studies (113-115). In some cases a decline in PPD from over 6 mm to 5 mm or less after conservative periodontal therapy eliminated the need for surgical treatment. In the PHMG-P group, PPD declined more rapidly, reaching its minimum by six months. Half a year later, PPD means in the CHX and water groups caught up with the values in the PHMG-P group.

Table 7. Average Pocket Probing Depth and reduction of periodontal pockets over 6 mm prior to and after treatment.

Treatment Group	PPD, mm (Mean±SD)					PPD reduction, mm (Mean±SD)				
	baseline	1 month	4 months	6 months	12 months	1 month	4 months	6 months	12 months	Total
PHMG-P	7.2±1.2	6.0±1.5	5.8±1.4	5.5±1.5	5.5±1.6	-1.2±1.3	-0.2±1.1	-0.2±1.1	0.02±0.9	-1.8±1.5
CHX	7.6±1.4	6.6±1.6	6.3±1.9	6.0±2.0	5.8±2.1	-1.0±1.2	-0.3±1.2	-0.3±1.1	-0.2±1.3	-1.7±1.9
Water	7.5±1.3	6.6±1.7	6.1±1.9	6.0±1.7	5.8±1.9	-0.9±1.2	-0.5±1.5	-0.2±1.3	-0.2±1.3	-1.7±1.7

At baseline, neither the total nor the average number of deep periodontal pockets (≥ 6 mm) per patient differed between treatment groups (Table 8). During the study, the number of pockets decreased significantly within the groups. No inter-group differences were observed at any follow-up examination. However, in the PHMG-P group, there was a pronounced tendency towards more rapid closure of deep periodontal pockets ($p>0.05$). By the end of the study, only patients in the antiseptic groups no longer required surgery. All deep periodontal pockets of two patients in the PHMG-P group and one patient in the CHX group healed below the threshold value for surgical intervention. Reduction in the depth of periodontal pockets reduces the need for surgery. However, a single patient with only one residual deep pocket still will require surgery, albeit not extensive. In the present study, all deep periodontal pockets of three patients in the antiseptic groups healed to the extent that periodontal surgery was no longer indicated. However, the paucity of data and the low number of clinical cases restrain any definite conclusions to be drawn from these results.

Table 8. Absolute and average number of deep (> 6 mm) periodontal pockets prior to and after treatment.

Treatment Group	Absolute number of deep periodontal pockets					Average number of deep periodontal pockets				
	baseline	1 month	4 months	6 months	12 months	baseline	1 month	4 months	6 months	12 months
PHMG-P	245	157	128	102	89	12.9±6.7	8.3±3.4	6.7±3.4	6.0±3.5	5.6±4.1
CHX	250	201	174	146	133	11.9±8.4	9.6±7.3	8.3±8.1	7.7±7.4	7.0±8.7
Water	243	206	179	169	152	12.8±11.0	10.8±9.4	9.4±8.0	9.4±8.2	8.4±8.2

4.8 Effect of PHMG-P irrigation on microflora and inflammatory markers in patients with periodontitis

With respect to both cytokine concentrations and the bacterial counts, there were no intergroup differences at any follow up ($p>0.05$). However, certain variations and distinctions in microbial composition and dynamics of biomarkers were recorded.

Total protein concentration followed a similar pattern in all the groups (Fig. 11). At the two-week time-point it decreased from the baseline value, then gradually increased up to 4 months. In the PHMG-P group the concentration of total protein increased from the baseline level, but in the CHX and water groups it remained below the initial titer. The decline of total protein amount in the CHX group was considerable ($p\leq 0.05$), whereas none of the changes observed in the PHMG-P and water groups during the study were significant ($p>0.05$).

IL-1 β production in PHMG-P and water groups exhibited a peak at the 2 week time-point and then declined below the initial level in case of PHMG-P and exceeded it in the water group. However, the changes were significant only in the PHMG-P group ($p\leq 0.05$) (Fig 11). In the CHX group the cytokine level declined constantly until the end of the first month and then increased slightly ($p>0.05$).

IL-8 is a chemokine, a chemoattractant for neutrophils (116). The dynamics of IL-8 concentration coincided with that of IL-1 β in the corresponding groups (Fig 11). The chemokine showed elevated values at the 2 week time-point in the PHMG-P and water groups, followed by a gradual decline by 4 months. In the CHX group there were negligible fluctuations in IL-8 production, which remained relatively constant. No differences in intragroup levels of IL-8 were recorded at any follow-up examination ($p>0.05$).

IL-10 regarded as an anti-inflammatory cytokine can be produced by many immune cells such as macrophages and regulatory T-cells. It affects dendritic cells, macrophages and several T-cell subsets (117). As an anti-inflammatory cytokine IL-10 downregulates the cellular immune response. It seems that production of IL-10 was synchronized with the dynamics of the pro-inflammatory cytokines (IL-1 β , IL-8 and IL-17A) to balance the strength of the immune response (Fig 11). No intragroup differences in IL-10 concentration were observed over the course of the study ($p>0.05$). Initially, the mean IL-10 level in the CHX group was about 3 times higher than in the other groups, but tended to decline with time. In the PHMG-P and water groups IL-10 increased from baseline to the 2 week time-point and then gradually declined by the 4 month time-point in the water group.

IL-17A is produced by T helper 17 subset of the T cell population (118). The receptor which binds IL-17A coordinates local tissue inflammation and up-regulates a wide range of pro-inflammatory cytokines and chemokines (119). The level of IL-17A decreased steadily in the CHX group and fluctuated significantly in the PHMG-P group and insignificantly in the water group (Fig 11).

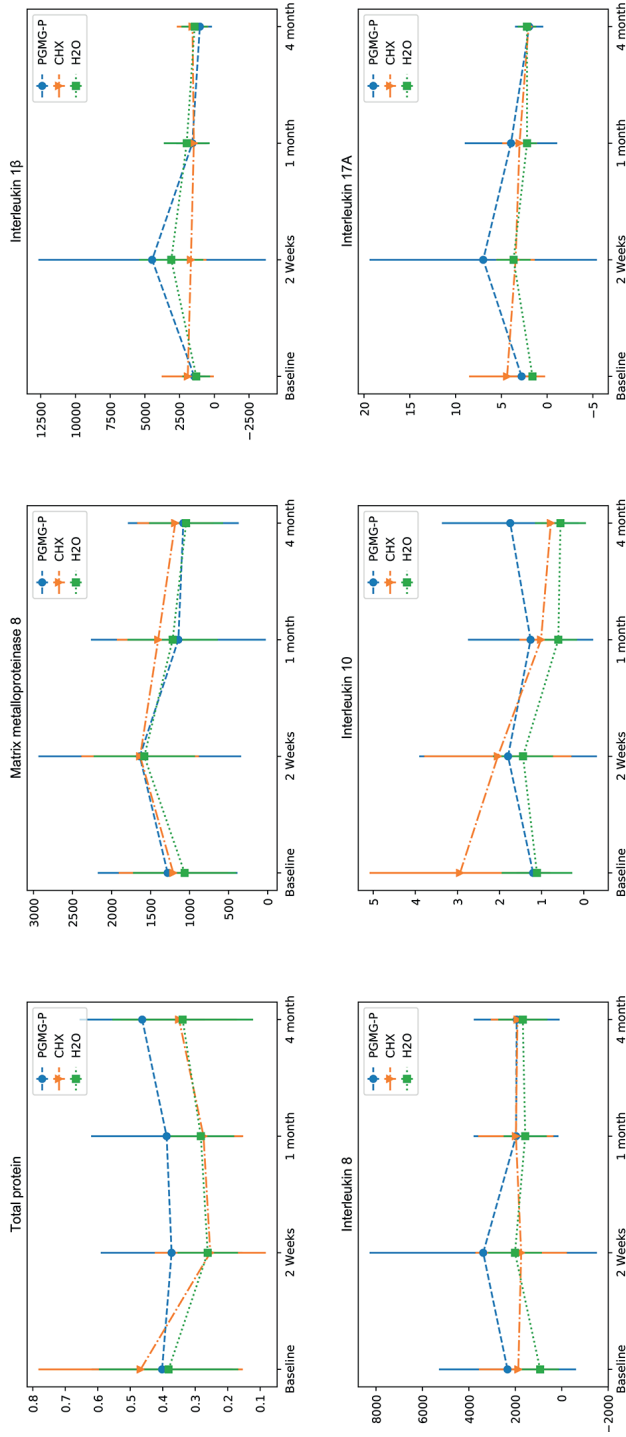


Figure 11. Dynamics of biomarkers in the course of the study.

During the study the concentration kinetics of **MMP-8** was not linear in any of the groups (Fig 11). At 2 week follow-up MMP-8 increased, then gradually declined to below the initial level. However, the changes were not significant ($p>0.05$). MMP-8 concentration varied between patients in test groups and the effect of the treatment was sometimes the opposite in different patients (data not shown).

On the average, *P. gingivalis* was detected in 70.2% of patients suffering from severe chronic periodontitis. In all treatment groups, the changes in concentration of *P. gingivalis* were similar throughout the study (Fig 12). It decreased gradually in the PHMG-P and CHX groups up to the one month time-point. In the water group the *P. gingivalis* titer dropped more sharply within the first fortnight. From 1 month to 4 months the bacterial count resumed an upward trend, but did not exceed the initial baseline value. The changes were significant only within the PHMG-P group ($p \leq 0.05$).

At baseline *T. denticola* was detected on the average in 68% of the patients. The concentration in the CHX group gradually decreased by 2-week and 1-month time-points, followed by a slight increase, yet it did not regain the baseline level. The changes were statistically significant ($p \leq 0.05$) (Fig 12). In patients treated with PHMG-P, *T. denticola* concentration dynamics showed an irregular pattern, increasing by the 2-week time-point then decreasing in 1 month, followed by regrowth exceeding the initial level by the 4-month checkpoint. In patients from the control group the concentration of *T. denticola* after short 2 weeks increase, it declined by 1 month. The changes in PHMG-P and water groups were not significant ($p > 0.05$).

In all three groups amount of *T. forsythia* (detected in 94.2% of total samples) decreased within a month and increased thereafter. However, the initial level was not regained (Fig 12). The changes became significant in the group treated with CHX ($p \leq 0.05$).

F. nucleatum was detected in every patient included in the study. Mechanical debridement and irrigation with CHX or PHMG-P decreased its concentration during the first month, but the initial level was almost regained by 4 months (Fig 12). Mechanical treatment along with flushing of periodontal pockets with water reduced the bacterial concentration at the 2-week follow-up, but further treatment had a limited effect and the concentration of *F. nucleatum* increased to approximately the original level.

A. actinomycetemcomitans (detected in 55.3% of total samples) titer rose during the first fortnight. Then *A. actinomycetemcomitans* count increased further in the water group, but decreased in the groups treated with antiseptics (Fig 12). Thereafter the *A. actinomycetemcomitans* count decreased to below the baseline level in all three groups. The changes within treatment groups were not significant ($p > 0.05$).

On the average *P. intermedia* was detected in 59.6% of the samples. The *P. intermedia* counts differed initially between subjects, but the differences were not statistically significant ($p > 0.05$) (Fig 12). During the study *P. intermedia* in treatment groups decreased insignificantly ($p > 0.05$). Mechanical debridement combined with water irrigation of the pockets cut concentration of microorganisms by 2 weeks, but they recovered by 1 month and further treatment reduced the *P. intermedia* count to levels comparable to the test groups.

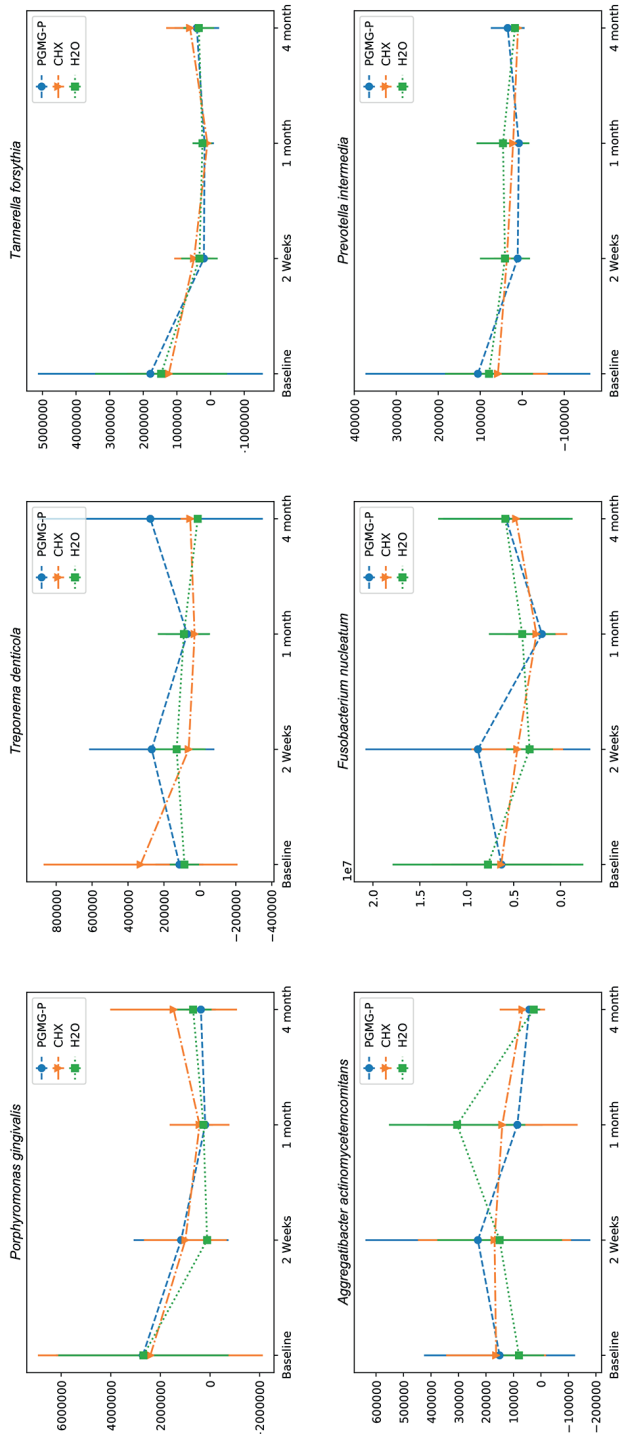


Figure 12. Dynamics of periopathogenic bacteria in the course of the study.

Although in the CHX group pro-inflammatory cytokines were not upregulated after 2 weeks, the increase in MMP-8 level in this group was similar to that in the PHMG-P and water groups. A rise in MMP-8 concentration at the 2 week follow-up after baseline treatment may be associated with mechanical injury of tissues during SRP and release of bacterial LPS. A further decrease in MMP-8 coincided with a reduction in PI and the ratios of *P. gingivalis*, *T. forsythia* and *P. intermedia*. In contrast to the 2 week follow-up, at the 1 month time-point, after the second treatment session, MMP-8 concentration did not increase. This may be accounted for by the effect of treatment and improved oral hygiene, a decrease in the number of microorganisms of the red and orange complexes and the LPS burden.

The dynamics of IL-1 β and MMP-8 were similar in the PHMG-P and water group, confirming previous findings that MMP-8 is controlled by the cytokine (120). The above-described *in vitro* study on the effects of CHX and PHMG-P on the secretion of inflammatory mediators by human gingival fibroblasts showed that CHX was able to arrest the accumulation of the pro-inflammatory cytokines IL-6, 8 and MMP-1 (121). In the present study CHX treatment, in contrast to PHMG-P, did not induce an upsurge of IL-1 β , IL-8 IL-17A level in subjects, suggesting that the cytokines are not the only factors regulating MMP-8 production.

At baseline the concentrations of IL-17A registered in subjects were diverse. The cytokine level was higher in the CHX group than in the other groups. Previous longitudinal studies have shown that biomarker levels could vary considerably, and that their response to therapy was not simple (122).

As previously reported, biomarker levels can exhibit considerable variability. In the present study diverse concentrations of cytokines were registered and this was reflected as high SD values as well as variegated response to therapy (122). In periodontal pathogenesis cytokines function in a complex network of overlapping intermolecular interactions, hence pathogenesis of periodontal disease is likely to be a far more complex process than previously assumed (21).

The decrease in *P. gingivalis*, *T. forsythia* and *P. intermedia* counts coincided with improvement of clinical parameters such as PI, BOP and PPD.

The reduction of *P. gingivalis* occurred at 2 weeks, but this was not accompanied by inhibition of the cytokines in the PHMG-P and water groups. Persistence of higher cytokine levels may be due to mechanical trauma during debridement, and the presence of LPS in the dental plaque, enhancing the original inflammatory response.

Alterations in the number of *P. gingivalis* and *T. forsythia* followed a similar pattern in all groups: growth inhibition up to 1 month (debridement every fortnight), and regrowth afterwards. Without active intervention the sites were re-populated by the bacteria.

The contrast in behavior of *A. actinomycetemcomitans* with that of the other bacteria might be explained by the fact that as some bacteria decreased, leaving vacant niches, *A. actinomycetemcomitans* proliferated and occupied these niches, and was displaced again by re-growth of other bacteria as the proportion of periodopathogens was restored.

It was observed that *F. nucleatum* count was restored to a greater extent than that of other bacteria. *F. nucleatum* is an essential microbe allowing anaerobes to bind to the plaque and facilitating biofilm development (123). The three month interval between treatment sessions was long enough for bacteria to repopulate the pockets.

The microbial count of every studied periodontopathogen in subgingival biofilm varied considerably between patients. This phenomenon of subject-to-subject heterogeneity in subgingival microbial profiles has been reported previously (124). The treatment displayed similar inhibiting effect on the tested bacteria, without statistically significant differences between treatment groups ($p>0.05$). In the same time changes in *T. forsythia*, *A. actinomycetemcomitans*, and *F. nucleatum* counts were significant within antiseptic groups ($p\leq 0.05$).

All studied microorganisms presumably played different roles in the development of inflammation. *P. gingivalis* and *T. forsythia* evidently played a key part, as all subjects carried heavy loads of these pathogens. The impact of CHX and PHMG-P on these microorganisms was similar ($p>0.05$). *F. nucleatum* behaved like an ordinary microbe: its amount fell during the treatment but regained approximately the original level afterwards. A decline in LPS concentration during treatment resulted in the arrest of inflammation and a decrease in pro-inflammatory cytokines, with eventual improvement of clinical parameters, specifically PPD, BOP, and PI (125).

5 CONCLUDING REMARKS

5.1 Summary of major findings

PHMG-P displayed anti-bacterial activity against standard quality control strains, periopathogens and *S. mutans*, even after a 20-fold dilution.

Both PHMG-P and CHX displayed cytotoxic effects towards fibroblasts at concentrations lower than those used in clinical practice. PHMG-P exhibited higher toxicity within a shorter time frame than CHX.

Low doses of PHMG-P and CHX reduced IL-1 β -induced secretion of PGE₂, IL-6, IL-8 and MMP-1 by gingival fibroblasts.

PHMG-P has a potential role in treatment of periodontal diseases. The studies confirm both its antimicrobial activity and the ability to lower concentrations of pro-inflammatory mediators.

Irrigation of periodontal pockets with PHMG-P according to the study protocol significantly reduced PPD in the short-term. However, by the end of this one-year trial, antiseptic irrigation had no long-term benefit on mean pocket depth.

High variability of inflammatory biomarkers and bacterial counts was observed. Antiseptic treatment caused changes in the biomarker concentrations and bacterial counts. The response to periodontal treatment was attributed mainly to mechanical debridement, while the contribution of adjunctive antiseptics, administered according to the protocol applied in these studies, was limited.

Overall conclusion:

Preclinical studies confirmed the antimicrobial and anti-inflammatory properties of PHMG-P. However, the subsequent one-year clinical trial disclosed that adjunctive irrigation with PHMG-P solution during scaling and root-planing had only a limited additional benefit: the therapeutic effect was attributable primarily to mechanical debridement.

5.2 Future perspectives

Based on the results of the studies presented in this thesis, further research is planned into the pharmacodynamics of antiseptics in periodontal pockets.

The following specific research aspects will be explored:

1. What concentration of antiseptic is retained in the periodontal pocket upon its delivery?
2. How long is the bactericidal concentration of antiseptics maintained at the site of administration?
3. How often antiseptic application procedure should be repeated?

A “split mouth experimental design” will be used. A major advantage of this technique is that the risk of interpersonal variation is circumvented and different treatments can be applied under the same conditions. The dental arch is divided into two equal parts, left and right. Each part is treated with different antiseptics (PHMG-P and CHX). Periodontal pockets will be rinsed with one of the antiseptics. The pocket contents will be aspirated immediately after administration and then after 0.5, 1, 3 and 5 min. The concentrations of antiseptics in the aspirated samples will be evaluated by mass spectrometry.

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7 REFERENCES

1. Caton JG, Armitage G, Berglundh T, Chapple ILC, Jepsen S, Kornman KS, et al. A new classification scheme for periodontal and peri-implant diseases and conditions - Introduction and key changes from the 1999 classification. *J Periodontol*. 2018;89 Suppl 1:S1-S8.
2. Durham J, Fraser HM, McCracken GI, Stone KM, John MT, Preshaw PM. Impact of periodontitis on oral health-related quality of life. *J Dent*. 2013;41(4):370-6.
3. Rydén L, Buhlin K, Ekstrand E, de Faire U, Gustafsson A, Holmer J, et al. Periodontitis Increases the Risk of a First Myocardial Infarction A Report From the PAROKRANK Study. *Circulation*. 2016;133(6):576-83.
4. Kinane DF, Stathopoulou PG, Papapanou PN. Periodontal diseases. *Nat Rev Dis Primers*. 2017;3:17038.
5. Eke PI, Dye BA, Wei L, Thornton-Evans GO, Genco RJ. Prevalence of periodontitis in adults in the United States: 2009 and 2010. *J Dent Res*. 2012;91(10):914-20.
6. Armitage GC. Development of a classification system for periodontal diseases and conditions. *Ann Periodontol*. 1999;4(1):1-6.
7. Theilade E. The non-specific theory in microbial etiology of inflammatory periodontal diseases. *J Clin Periodontol*. 1986;13(10):905-11.
8. Loesche WJ, Grossman NS. Periodontal disease as a specific, albeit chronic, infection: diagnosis and treatment. *Clin Microbiol Rev*. 2001;14(4):727-52, table of contents.
9. Haffajee AD, Socransky SS. Microbial etiological agents of destructive periodontal diseases. *Periodontol 2000*. 1994;5:78-111.
10. Haffajee A, Teles R, Socransky S. Association of *Eubacterium nodatum* and *Treponema denticola* with human periodontitis lesions. *Oral Microbiol Immunol*. 2006;21(5):269-82.
11. Teles RP, Haffajee AD, Socransky SS. Microbiological goals of periodontal therapy. *Periodontol 2000*. 2006;42(1):180-218.
12. Marsh PD. Dental plaque: biological significance of a biofilm and community life-style. *J Clin Periodontol*. 2005;32 Suppl 6:7-15.
13. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science*. 1999;284(5418):1318-22.

14. Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. *Lancet*. 2001;358(9276):135-8.
15. Eick S, Selmann T, Pfister W. Efficacy of antibiotics to strains of periodontopathogenic bacteria within a single species biofilm - an in vitro study. *J Clin Periodontol*. 2004;31(5):376-83.
16. Sbordone L, Ramaglia L, Gulletta E, Iacono V. Recolonization of the subgingival microflora after scaling and root planing in human periodontitis. *J Periodontol*. 1990;61(9):579-84.
17. Renvert S, Pettersson T, Ohlsson O, Persson GR. Bacterial profile and burden of periodontal infection in subjects with a diagnosis of acute coronary syndrome. *J Periodontol*. 2006;77(7):1110-9.
18. Cochran DL. Inflammation and bone loss in periodontal disease. *J Periodontol*. 2008;79(8 Suppl):1569-76.
19. Garlet GP. Destructive and protective roles of cytokines in periodontitis: a re-appraisal from host defense and tissue destruction viewpoints. *J Dent Res*. 2010;89(12):1349-63.
20. Liu YC, Lerner UH, Teng YT. Cytokine responses against periodontal infection: protective and destructive roles. *Periodontol 2000*. 2010;52(1):163-206.
21. Preshaw PM, Taylor JJ. How has research into cytokine interactions and their role in driving immune responses impacted our understanding of periodontitis? *J Clin Periodontol*. 2011;38 Suppl 11:60-84.
22. Van Dyke TE, van Winkelhoff AJ. Infection and inflammatory mechanisms. *J Clin Periodontol*. 2013;40 Suppl 14:S1-7.
23. Ara T, Kurata K, Hirai K, Uchihashi T, Uematsu T, Imamura Y, et al. Human gingival fibroblasts are critical in sustaining inflammation in periodontal disease. *J Periodontol Res*. 2009;44(1):21-7.
24. Mahanonda R, Pichyangkul S. Toll-like receptors and their role in periodontal health and disease. *Periodontol 2000*. 2007;43:41-55.
25. Graves DT, Li J, Cochran DL. Inflammation and uncoupling as mechanisms of periodontal bone loss. *J Dent Res*. 2011;90(2):143-53.
26. Behl Y, Siqueira M, Ortiz J, Li J, Desta T, Faibish D, et al. Activation of the acquired immune response reduces coupled bone formation in response to a periodontal pathogen. *J Immunol*. 2008;181(12):8711-8.

27. Beklen A, Ainola M, Hukkanen M, Gurgan C, Sorsa T, Konttinen YT. MMPs, IL-1, and TNF are regulated by IL-17 in periodontitis. *J Dent Res*. 2007;86(4):347-51.
28. Han X, Lin X, Seliger AR, Eastcott J, Kawai T, Taubman MA. Expression of receptor activator of nuclear factor-kappaB ligand by B cells in response to oral bacteria. *Oral Microbiol Immunol*. 2009;24(3):190-6.
29. Pestka S, Krause CD, Sarkar D, Walter MR, Shi Y, Fisher PB. Interleukin-10 and related cytokines and receptors. *Annu Rev Immunol*. 2004;22:929-79.
30. Bluestone JA, Mackay CR, O'Shea JJ, Stockinger B. The functional plasticity of T cell subsets. *Nat Rev Immunol*. 2009;9(11):811-6.
31. Funk CD. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science*. 2001;294(5548):1871-5.
32. Harris SG, Padilla J, Koumas L, Ray D, Phipps RP. Prostaglandins as modulators of immunity. *Trends Immunol*. 2002;23(3):144-50.
33. Noguchi K, Ishikawa I. The roles of cyclooxygenase-2 and prostaglandin E2 in periodontal disease. *Periodontol*. 2000;43:85-101.
34. Ide M, Papapanou PN. Epidemiology of association between maternal periodontal disease and adverse pregnancy outcomes--systematic review. *J Clin Periodontol*. 2013;40 Suppl 14:S181-94.
35. Dietrich T, Sharma P, Walter C, Weston P, Beck J. The epidemiological evidence behind the association between periodontitis and incident atherosclerotic cardiovascular disease. *J Clin Periodontol*. 2013;40 Suppl 14:S70-84.
36. Buhlin K, Hultin M, Norderyd O, Persson L, Pockley AG, Pussinen PJ, et al. Periodontal treatment influences risk markers for atherosclerosis in patients with severe periodontitis. *Atherosclerosis*. 2009;206(2):518-22.
37. Taylor JJ, Preshaw PM, Lalla E. A review of the evidence for pathogenic mechanisms that may link periodontitis and diabetes. *J Clin Periodontol*. 2013;40 Suppl 14:S113-34.
38. Linden GJ, Herzberg MC, Working group 4 of joint EFPAAPw. Periodontitis and systemic diseases: a record of discussions of working group 4 of the Joint EFP/AAP Workshop on Periodontitis and Systemic Diseases. *J Clin Periodontol*. 2013;40 Suppl 14:S20-3.
39. Linden GJ, Linden K, Yarnell J, Evans A, Kee F, Patterson CC. All-cause mortality and periodontitis in 60-70-year-old men: a prospective cohort study. *J Clin Periodontol*. 2012;39(10):940-6.

40. Quirynen M, Bollen CM, Vandekerckhove BN, Dekeyser C, Papaioannou W, Eyssen H. Full- vs. partial-mouth disinfection in the treatment of periodontal infections: short-term clinical and microbiological observations. *J Dent Res*. 1995;74(8):1459-67.
41. Quirynen M, Mongardini C, Pauwels M, Bollen CM, Van Eldere J, van Steenberghe D. One stage full- versus partial-mouth disinfection in the treatment of chronic adult or generalized early-onset periodontitis. II. Long-term impact on microbial load. *J Periodontol*. 1999;70(6):646-56.
42. Apatzidou DA, Kinane DF. Quadrant root planing versus same-day full-mouth root planing. *J Clin Periodontol*. 2004;31(2):132-40.
43. Swierkot K, Nonnenmacher CI, Mutters R, Flores-De-Jacoby L, Mengel R. One-stage full-mouth disinfection versus quadrant and full-mouth root planing. *J Clin Periodontol*. 2009;36(3):240-9.
44. The American Academy of Periodontology Statement Regarding Gingival Curettage. *J Periodontol*. 2002;73(10):1229-30.
45. Waerhaug J. Effect of rough surfaces upon gingival tissue. *J Dent Res*. 1956;35(2):323-5.
46. Lang NP. Focus on intrabony defects--conservative therapy. *Periodontol* 2000. 2000;22:51-8.
47. Nyman S, Westfelt E, Sarhed G, Karring T. Role of "diseased" root cementum in healing following treatment of periodontal disease. A clinical study. *J Clin Periodontol*. 1988;15(7):464-8.
48. Hanes PJ, Purvis JP. Local anti-infective therapy: pharmacological agents. A systematic review. *Ann Periodontol*. 2003;8(1):79-98.
49. Perinetti G, Paolantonio M, Cordella C, D'Ercole S, Serra E, Piccolomini R. Clinical and microbiological effects of subgingival administration of two active gels on persistent pockets of chronic periodontitis patients. *J Clin Periodontol*. 2004;31(4):273-81.
50. Bjarnsholt T, Kirketerp-Moller K, Jensen PO, Madsen KG, Phipps R, Krogfelt K, et al. Why chronic wounds will not heal: a novel hypothesis. Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society. 2008;16(1):2-10.
51. Anoop K, Ranjan M, Vishakha G, Deepak G. Systemic antibiotic therapy in periodontics. *Dent Res J*. 2012;9(5):505-15.

52. Cionca N, Giannopoulou C, Ugolotti G, Mombelli A. Amoxicillin and metronidazole as an adjunct to full-mouth scaling and root planing of chronic periodontitis. *J Periodontol*. 2009;80(3):364-71.
53. Herrera D, Alonso B, Leon R, Roldan S, Sanz M. Antimicrobial therapy in periodontitis: the use of systemic antimicrobials against the subgingival biofilm. *J Clin Periodontol*. 2008;35(8 Suppl):45-66.
54. McDonnell G, Russell AD. Antiseptics and disinfectants: activity, action, and resistance. *Clin Microbiol Rev*. 1999;12(1):147-79.
55. Weinberger BW. An introduction to the history of dentistry: Mosby ST Louis; 1948.
56. Fischman SL. The history of oral hygiene products: how far have we come in 6000 years? *Periodontol 2000*. 1997;15:7-14.
57. Jardim JJ, Alves LS, Maltz M. The history and global market of oral home-care products. *Braz Oral Res*. 2009;23 Suppl 1:17-22.
58. Johnson & Johnson Consumer Inc. History of LISTERINE®: From Surgery Antiseptic to Modern Mouthwash.
59. Davies GE, Francis J, Martin AR, Rose FL, Swain G. 1:6-Di-4'-chlorophenyldiguanidohexane (hibitane); laboratory investigation of a new antibacterial agent of high potency. *Br J Pharmacol Chemother*. 1954;9(2):192-6.
60. Gjermo P. Chlorhexidine in dental practice. *J Clin Periodontol*. 1974;1(3):143-52.
61. Jones CG. Chlorhexidine: is it still the gold standard? *Periodontol 2000*. 1997;15:55-62.
62. Kuyyakanond T, Quesnel LB. The mechanism of action of chlorhexidine. *FEMS Microbiol Lett*. 1992;100(1-3):211-5.
63. Mariotti AJ, Rumpf DA. Chlorhexidine-induced changes to human gingival fibroblast collagen and non-collagen protein production. *Journal of periodontology*. 1999;70(12):1443-8.
64. Giannelli M, Chellini F, Margheri M, Tonelli P, Tani A. Effect of chlorhexidine digluconate on different cell types: a molecular and ultrastructural investigation. *Toxicol In Vitro*. 2008;22(2):308-17.
65. Bonito AJ, Lux L, Lohr KN. Impact of local adjuncts to scaling and root planing in periodontal disease therapy: a systematic review. *J Periodontol*. 2005;76(8):1227-36.

66. Guarnelli ME, Franceschetti G, Manfrini R, Trombelli L. Adjunctive effect of chlorhexidine in ultrasonic instrumentation of aggressive periodontitis patients: a pilot study. *J Clin Periodontol.* 2008;35(4):333-41.
67. Schenkein HA, Loos BG. Inflammatory mechanisms linking periodontal diseases to cardiovascular diseases. *J Clin Periodontol.* 2013;40 Suppl 14:S51-69.
68. Sahrman P, Manz A, Attin T, Zbinden R, Schmidlin PR. Effect of application of a PVP-iodine solution before and during subgingival ultrasonic instrumentation on post-treatment bacteraemia: a randomized single-centre placebo-controlled clinical trial. *J Clin Periodontol.* 2015;42(7):632-9.
69. Rosin M, Welk A, Bernhardt O, Ruhnau M, Pitten FA, Kocher T, et al. Effect of a polyhexamethylene biguanide mouthrinse on bacterial counts and plaque. *J Clin Periodontol.* 2001;28(12):1121-6.
70. Ikeda T, Ledwith A, Bamford CH, Hann RA. Interaction of a polymeric biguanide biocide with phospholipid membranes. *Biochim Biophys Acta.* 1984;769(1):57-66.
71. Gilbert P, Moore LE. Cationic antiseptics: diversity of action under a common epithet. *J Appl Microbiol.* 2005;99(4):703-15.
72. Allen MJ, Morby AP, White GF. Cooperativity in the binding of the cationic biocide polyhexamethylene biguanide to nucleic acids. *Biochem Biophys Res Commun.* 2004;318(2):397-404.
73. Rosin M, Welk A, Kocher T, Majic-Todt A, Kramer A, Pitten FA. The effect of a polyhexamethylene biguanide mouthrinse compared to an essential oil rinse and a chlorhexidine rinse on bacterial counts and 4-day plaque regrowth. *J Clin Periodontol.* 2002;29(5):392-9.
74. Welk A, Splieth CH, Schmidt-Martens G, Schwahn C, Kocher T, Kramer A, et al. The effect of a polyhexamethylene biguanide mouthrinse compared with a triclosan rinse and a chlorhexidine rinse on bacterial counts and 4-day plaque re-growth. *J Clin Periodontol.* 2005;32(5):499-505.
75. Muller G, Kramer A. Biocompatibility index of antiseptic agents by parallel assessment of antimicrobial activity and cellular cytotoxicity. *J Antimicrob Chemother.* 2008;61(6):1281-7.
76. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods.* 1983;65(1-2):55-63.
77. Hanemaaijer R, Sorsa T, Konttinen YT, Ding Y, Sutinen M, Visser H, et al. Matrix metalloproteinase-8 is expressed in rheumatoid synovial fibroblasts

- and endothelial cells. Regulation by tumor necrosis factor-alpha and doxycycline. *J Biol Chem.* 1997;272(50):31504-9.
78. Gursoy UK, Kononen E, Pradhan-Palikhe P, Tervahartiala T, Pussinen PJ, Suominen-Taipale L, et al. Salivary MMP-8, TIMP-1, and ICTP as markers of advanced periodontitis. *J Clin Periodontol.* 2010;37(6):487-93.
 79. Yoshida A, Nagashima S, Ansai T, Tachibana M, Kato H, Watari H, et al. Loop-mediated isothermal amplification method for rapid detection of the periodontopathic bacteria *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*. *J Clin Microbiol.* 2005;43(5):2418-24.
 80. Boutaga K, van Winkelhoff AJ, Vandenbroucke-Grauls CM, Savelkoul PH. Periodontal pathogens: a quantitative comparison of anaerobic culture and real-time PCR. *FEMS Immunol Med Microbiol.* 2005;45(2):191-9.
 81. Hyvarinen K, Laitinen S, Paju S, Hakala A, Suominen-Taipale L, Skurnik M, et al. Detection and quantification of five major periodontal pathogens by single copy gene-based real-time PCR. *Innate Immun.* 2009;15(4):195-204.
 82. Coffey J, Choudhry M, Shlossman M, Makin IRS, Singh VK. Multiplex real-time PCR detection and relative quantification of periodontal pathogens. *Clin Exp Dent Res.* 2016;2(3):185-92.
 83. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* 2003;31(13):3406-15.
 84. The RNA Institute. College of Arts and Sciences UAA, State University of New York,. DNA folding form.
 85. Sanchez M, Llama-Palacios A, Fernandez E, Figuero E, Marin M, Leon R, et al. An in vitro biofilm model associated to dental implants: structural and quantitative analysis of in vitro biofilm formation on different dental implant surfaces. *Dent Mater.* 2014;30(10):1161-71.
 86. Koressaar T, Remm M. Enhancements and modifications of primer design program Primer3. *Bioinformatics.* 2007;23(10):1289-91.
 87. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3--new capabilities and interfaces. *Nucleic Acids Res.* 2012;40(15):e115.
 88. Whitehead Institute for Biomedical Research. Primer3. 2019.
 89. Lang NP, Lindhe J. Clinical periodontology and implant dentistry, 2 Volume Set: John Wiley & Sons; 2015.
 90. Umland TC, Schultz LW, MacDonald U, Beanan JM, Olson R, Russo TA. In vivo-validated essential genes identified in *Acinetobacter baumannii* by

using human ascites overlap poorly with essential genes detected on laboratory media. *mBio*. 2012;3(4).

91. Krasil'nikov AP, Adarchenko AA. [The clinical significance and methodological problems in determining bacterial sensitivity/resistance to antiseptics]. *Antibiotiki i khimioterapiia* = Antibiotics and chemotherapy [sic] / Ministerstvo meditsinskoi i mikrobiologicheskoi promyshlennosti SSSR. 1992;37(9):39-44.
92. Vitt A, Sofrata A, Slizen V, Sugars RV, Gustafsson A, Gudkova EI, et al. Antimicrobial activity of polyhexamethylene guanidine phosphate in comparison to chlorhexidine using the quantitative suspension method. *Ann Clin Microbiol Antimicrob*. 2015;14:36.
93. Rathbone MJ, Tucker IG. Mechanisms, barriers and pathways of oral mucosal drug permeation. *Advanced Drug Delivery Reviews*. 1993;12(1-2):41-60.
94. Pantos A, Tsogas I, Paleos CM. Guanidinium group: a versatile moiety inducing transport and multicompartimentalization in complementary membranes. *Biochimica et Biophysica Acta (BBA)-Biomembranes*. 2008;1778(4):811-23.
95. Hwang HJ, Nam J, Yang SI, Kwon J-H, Oh HB. MALDI-TOF Analysis of Polyhexamethylene Guanidine (PHMG) Oligomers Used as a Commercial Antibacterial Humidifier Disinfectant. *Bulletin of the Korean Chemical Society*. 2013;34(6):1708-14.
96. Hong SB, Kim HJ, Huh JW, Do KH, Jang SJ, Song JS, et al. A cluster of lung injury associated with home humidifier use: clinical, radiological and pathological description of a new syndrome. *Thorax*. 2014;69(8):694-702.
97. Kim HR, Hwang GW, Naganuma A, Chung KH. Adverse health effects of humidifier disinfectants in Korea: lung toxicity of polyhexamethylene guanidine phosphate. *J Toxicol Sci*. 2016;41(6):711-7.
98. Kim HR, Lee K, Park CW, Song JA, Shin DY, Park YJ, et al. Polyhexamethylene guanidine phosphate aerosol particles induce pulmonary inflammatory and fibrotic responses. *Arch Toxicol*. 2016;90(3):617-32.
99. Lee J, Jeong JS, Kim SY, Im WJ, Shin YJ, Lee K, et al. Reproductive and developmental toxicity screening of polyhexamethylene guanidine phosphate by oral gavage in rats. *Regul Toxicol Pharmacol*. 2019;108:104440.
100. Kramer A, Eberlein T, Müller G, Dissemmond J, Assadian O. Re-evaluation of polyhexanide use in wound antisepsis in order to clarify ambiguities of two animal studies. *J Wound Care*. 2019;28(4):246-55.

101. Lee JH, Yu IJ. Human exposure to polyhexamethylene guanidine phosphate from humidifiers in residential settings: Cause of serious lung disease. *Toxicol Ind Health*. 2017;33(11):835-42.
102. Lee E, Son SK, Yoon J, Cho HJ, Yang SI, Jung S, et al. Two Cases of Chloromethylisothiazolinone and Methylisothiazolinone-associated Toxic Lung Injury. *J Korean Med Sci*. 2018;33(16):e119.
103. Dissemmond J, Gerber V, Kramer A, Riepe G, Strohal R, Vassel-Biergans A, et al. A practice-oriented recommendation for treatment of critically colonised and locally infected wounds using polyhexanide. *J Tissue Viability*. 2010;19(3):106-15.
104. Kratzer C, Tobudic S, Assadian O, Buxbaum A, Graninger W, Georgopoulos A. Validation of AKACID plus as a room disinfectant in the hospital setting. *Appl Environ Microbiol*. 2006;72(6):3826-31.
105. Buxbaum A, Kratzer C, Graninger W, Georgopoulos A. Antimicrobial and toxicological profile of the new biocide Akacid plus. *J Antimicrob Chemother*. 2006;58(1):193-7.
106. Unal N, Yanik K, Karadag A, Odabaşı H, Esen S, Günaydin M. Evaluation of the efficacy of akacid plus® fogging in eradicating causative microorganism in nosocomial infections. *Int J Clin Exp Med*. 2014;7(12):5867-71.
107. Kratzer C, Tobudic S, Macfelda K, Graninger W, Georgopoulos A. In vivo activity of a novel polymeric guanidine in experimental skin infection with methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother*. 2007;51(9):3437-9.
108. Neuwirt H, Wabnig E, Feistritzer C, Eder IE, Salvador C, Puhr M, et al. Akacid medical formulation induces apoptosis in myeloid and lymphatic leukemic cell lines in vitro and in vivo. *PLoS One*. 2015;10(2):e0117806.
109. Rohner E, Hoff P, Gaber T, Lang A, Voros P, Buttgereit F, et al. Cytokine expression in human osteoblasts after antiseptic treatment: a comparative study between polyhexanide and chlorhexidine. *J Invest Surg*. 2015;28(1):1-7.
110. Sakaki H, Matsumiya T, Kusumi A, Imaizumi T, Satoh H, Yoshida H, et al. Interleukin-1 β induces matrix metalloproteinase-1 expression in cultured human gingival fibroblasts: role of cyclooxygenase-2 and prostaglandin E₂. *Oral Dis*. 2004;10(2):87-93.
111. Turkoglu O, Becerik S, Emingil G, Kutukculer N, Baylas H, Atilla G. The effect of adjunctive chlorhexidine mouthrinse on clinical parameters and gingival crevicular fluid cytokine levels in untreated plaque-associated gingivitis. *Inflamm Res*. 2009;58(5):277-83.

112. Goodson JM. Gingival crevice fluid flow. *Periodontol* 2000. 2003;31:43-54.
113. Morrison EC, Ramfjord SP, Hill RW. Short-term effects of initial, nonsurgical periodontal treatment (hygienic phase). *J Clin Periodontol*. 1980;7(3):199-211.
114. Hammerle CH, Joss A, Lang NP. Short-term effects of initial periodontal therapy (hygienic phase). *J Clin Periodontol*. 1991;18(4):233-9.
115. Cobb CM. Clinical significance of non-surgical periodontal therapy: an evidence-based perspective of scaling and root planing. *J Clin Periodontol*. 2002;29 Suppl 2:6-16.
116. Souza PP, Lerner UH. The role of cytokines in inflammatory bone loss. *Immunological investigations*. 2013;42(7):555-622.
117. Banchereau J, Pascual V, O'Garra A. From IL-2 to IL-37: the expanding spectrum of anti-inflammatory cytokines. *Nat Immunol*. 2012;13(10):925-31.
118. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, et al. Interleukin 17-producing CD4⁺ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol*. 2005;6(11):1123-32.
119. Xu S, Cao X. Interleukin-17 and its expanding biological functions. *Cell Mol Immunol*. 2010;7(3):164-74.
120. Sorsa T, Tjaderhane L, Konttinen YT, Lauhio A, Salo T, Lee HM, et al. Matrix metalloproteinases: contribution to pathogenesis, diagnosis and treatment of periodontal inflammation. *Ann Med*. 2006;38(5):306-21.
121. Vitt A, Slizen V, Bostrom EA, Yucel-Lindberg T, Kats A, Sugars RV, et al. Effects of polyhexamethylene guanidine phosphate on human gingival fibroblasts. *Acta Odontol Scand*. 2017;75(7):524-9.
122. Teles FR, Teles RP, Martin L, Socransky SS, Haffajee AD. Relationships among interleukin-6, tumor necrosis factor-alpha, adipokines, vitamin D, and chronic periodontitis. *J Periodontol*. 2012;83(9):1183-91.
123. Kolenbrander PE, London J. Adhere today, here tomorrow: oral bacterial adherence. (Minireview). *J Bacteriol*. 1993;175(11 12):3247.
124. Kolenbrander PE, Palmer RJ, Jr., Rickard AH, Jakubovics NS, Chalmers NI, Diaz PI. Bacterial interactions and successions during plaque development. *Periodontol* 2000. 2006;42:47-79.
125. Vitt A, Gustafsson A, Ramberg P, Slizen V, Kazeko LA, Buhlin K. Polyhexamethylene guanidine phosphate irrigation as an adjunctive to scaling and root planing in the treatment of chronic periodontitis. *Acta Odontol Scand*. 2019:1-6.